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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

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Meetings are held at different centres for the communication of papers to the Society.

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OBITUARY NOTICES.

FREDERICK WILLIAM PAVY.

Dr Pavy was born at Wroughton in Wiltshire on May 29th, 1829. He received his preliminary education at the Merchant Taylors' School and entered Guy's Hospital in 1847. After five years, he graduated at the University of London, taking the M.B. degree with honours in all subjects and the gold medal in medicine. In the following year he obtained the M.D. degree, and then went to Paris, where he met Claude Bernard, for whom, in after life, he always had the greatest admiration although disagreeing in so pronounced a manner with his teaching. On his return to England he was appointed a lecturer of anatomy at Guy's Hospital in 1854, and lecturer in physiology in 1856, which latter position he held until 1877. In 1859 he became assistant physician at Guy's, and full physician in 1871, when he was also appointed lecturer in medicine at the medical school. On his retirement from active work at Guy's in 1890, he was appointed consulting physician to the Hospital.

Dr Pavy took an active part in the proceedings of the Royal College of Physicians, at which he was sometime examiner, censor and councillor. He was President of the Royal Medical and Chirurgical Society in 1893 and President of the Association for the Advancement of Scientific Research and Chairman of the National Committee of International Medical Congresses until his death on Sept. 19th, 1911. During his career he delivered the Lettsomian Lectures in 1859, the Goulstonian Lectures in 1862, the Croonian Lectures in 1878 and again in 1894, and was Harveian Orator of the Medical Society in 1886. He was elected a Fellow of the Royal Society in 1863.

In 1901 he was awarded the Baly Gold Medal by the Royal College of Physicians for his excellent physiological investigations, and, in celebration of his 80th birthday in 1909, the members of the Physiological Society presented him with a silver bowl in token of their affection for him and admiration of his work. Many other honours were awarded to him not only by his colleagues in this country but by the wider medical fellowship in all parts of the world. He achieved a high reputation for the treatment of diabetes in Europe and America whilst still comparatively young, and his intense vitality enabled

him to maintain a commanding position for more than fifty years as an expert in the department which he had made his own.

Although consulted daily by patients from all parts of the world, Dr Pavy was much more than a physician. When the writer was first brought into contact with him towards the end of his active career, he seemed to be the ideal research worker, bending all his energies to search out the solutions of the problems with which he was so familiar. The mornings were given up to consultations, when he was brought into direct contact with the anomalies which reveal the essentially complex nature of the phenomena which are comprised under the term diabetes; the afternoons were devoted to research work, and the evenings to a consideration of the daily results and to the drawing-up of the programme for the next day's experiments. He seemed to be always picturing in his mind the possible sequences of events in the body. Possessed of a remarkably vivid imagination he could *see* the molecules of starchy food being broken down in the alimentary canal into glucose molecules and could watch these simple molecules passing through the walls of the intestines and making their way to the liver, the muscles or the urine. From the visualised processes, deductions were drawn which he sought to substantiate or refute by the test of experiment. He was exceedingly careful, desiring accuracy in his research work and certainty in the deductions to be drawn from it. For him, the positive result of a single experiment was never sufficient to ground a theory on, and he was always on the look-out for the pitfalls which were a cause of stumbling to so many of his co-workers.

The knowledge obtained through long years of experience of the manifold errors by which workers in his field of research were so liable to be led astray impelled him to give but little consideration to contemporary literature. He sought above all else to express his own views in the clearest possible manner, and the only two books he consulted frequently were a large dictionary of several volumes and Roget's *Thesaurus*. The present writer remembers many lengthy discussions over the relative merits of certain kindred words and their ability to express the exact shade of meaning he desired to convey. His writings are models of careful expression and often took weeks and months to prepare. One result of this devotion to accuracy of expression was that he ascribed the same virtue to his contemporaries and often read into their words meanings which the authors themselves did not wish to convey. More than one of the controversies in which he took part arose in this way.

The majority of Dr Pavy's scientific publications are concerned with the metabolism of the carbohydrates in one or other of its aspects. His views on this subject changed very considerably with the passing years. In his early days he discovered that the glycogenic doctrine promulgated by Claude Bernard, which assigns to the liver a regulating function over the supply of carbohydrate to the tissues, was based on faulty experiments. He showed that the saccharine condition of the liver was due to *post-mortem* changes and was absent in the living animal. Further, according to his careful analyses, arterial blood contained only the same amount of dextrose as venous blood, which seemed to prove that dextrose did not pass from the liver to the tissues as free dextrose. Finally he maintained that the kidney acted as a filter which allowed all the diffusible substances in the blood, such as urea and dextrose, to pass into the urine, retaining only the indiffusible proteins within the blood-vessels. "Hence," he argues (in *Carbohydrate Metabolism and Diabetes*, 1906), "if sugar (dextrose) passed as free sugar into the circulation from the food or from the liver, as is contended for under the glycogenic doctrine, the urine could not fail to afford a revelation of the fact." His antagonism to the glycogenic doctrine was thus maintained to the end of his life, although his later experiments convinced him that the glycogen of the liver, after being broken down by enzyme action into dextrose, is normally transported by the blood to the tissues. He could not admit, however, that this passage was in the form of free dextrose, but thought that the dextrose must be adsorbed by, or otherwise loosely combined with, the proteins of the blood.

Dr Pavy regarded the blood as a food of constant composition for the tissue cells. Assimilation occurred at the seat of absorption in the intestines or in the liver, and the work of building-up protein from dextrose and amino-acids was considered by him to be done by the lymphocytes, whilst the transformation of the excess of dextrose into fat and glycogen took place in the columnar epithelium of the mucous membrane of the intestinal wall, and in the liver. Synthesis of protein by the growth of lymphocytes, like yeast cells, on the products of digestion in the wall of the alimentary canal is a view that appeals to the imagination; but it has not met with much favour from the sober-minded physiologists of the present day. The fat-forming powers of the columnar epithelial cells have also been denied, but the exact location of the transformation, which undoubtedly does occur in the body, is still unknown, and it may be that the future will see the vindication of this as well as of other visions of Dr Pavy.

Perhaps the most startling of his discoveries in the domain of biochemistry was the recognition of the carbohydrate groups in the protein molecule. Dr Pavy's theory of the glucosidic nature of the proteins was received with interest throughout the scientific world, and a multiplicity of gluco-proteins have since been isolated. Later investigations have shown, however, that the carbohydrate linkage is not of a glucosidic nature so that the metabolic value assigned to these groups by Dr Pavy has not been generally accepted although no other explanation of their significance has yet been attempted.

Among his contributions to chemical physiology must also be mentioned the method for the quantitative estimation of reducing sugar by means of Pavy's ammoniated cupric test which affords excellent results after a little practice.

In the treatment of diabetes, Dr Pavy did much to provide a more suitable and palatable dietary for the diabetic, and, indeed, was responsible for the introduction of almond and other protein-rich bread substitutes. Regarding excess of free dextrose in the blood of diabetics as exerting a toxic action on the system, it seemed to him essential to stop its flow through the body and his treatment usually commenced by placing the patient on a carbohydrate-free diet. Afterwards, as assimilative power returned, starchy food was allowed, but only in such quantity as to maintain the urine free from dextrose. He was among the first to grasp the importance of the acetone series of substances eliminated especially in acute stages of the disease, and published several papers concerning them. The albuminuria often associated with diabetes naturally interested him as well as the cyclic or physiological albuminuria which he was the first to recognise.

Although many of his views are unorthodox and may be considered improbable, Dr Pavy's critical spirit and his unflagging enthusiasm for experimental research have exerted a stimulating influence on two generations of physiologists, and he has thus been the means, indirectly as well as directly, of extending the borders of our knowledge of carbohydrate metabolism and diabetes. His memory will be cherished by all who were privileged to be brought into intimate relations with him, and his example will be an inspiration throughout all future days.

Dr Pavy married in 1855 Julia, daughter of Mr William Oliver, by whom he had a daughter, Florence, who married the Rev. Borradaile Savory, son of the famous surgeon, Sir William Savory, and father of the present baronet.

H. W. Bywaters.

JOHN WADE.

JOHN WADE, though not a biochemist by title, or indeed by occupation, had the keenest sympathy with all who seek to apply chemistry to the problems of the plant and animal. He was greatly interested in the foundation of the Biochemical Society. During the most active part of his life Wade taught chemistry to medical students, and, unlike some who perform the same duty, he was not content to deal with the subject in a watertight compartment. He had himself no practical knowledge of medicine, but he made it his business to discover, so far as he could, what were the chemical needs of contemporary pathology and clinical medicine, and saw to it that his teaching, in spite of this or that unwise examination schedule, should have some bearing on those needs. He was, it is true, oddly but obstinately convinced that there was something in the nature of biological problems which put them beyond the grasp of his mind, and he was undoubtedly averse from doing any kind of experimental work which did not employ methods of the highest accuracy. Otherwise I think he would have been found among the pioneers of biochemistry, for he was convinced of its outstanding importance. It was perhaps unfortunate for our own particular science that his work was done on other lines, for Wade was a man possessed of great scientific foresight and was a chemist of genius.

He was born at Deptford, on Jan. 13th, 1864. At the early age of 16 he entered the private laboratory of Dr (afterwards Sir Thomas) Stevenson at Guy's Hospital, to act, more or less informally, as a junior assistant. Although this start in life appeared to mark him for a chemist, it was really only long afterwards that Wade definitely chose his career. Not until he was 27 years of age and received the offer of the post of Senior Demonstrator in the Medical School of Guy's Hospital was his decision finally made. Only then did his mind really come to grips with organic chemistry—the subject which he was afterwards to know so intimately and teach so admirably. On his appointment at Guy's he set to work to organise the practical teaching in the new medical school laboratories on original and stimulating lines, and when he felt that this task had been accomplished he began to gather material for his textbook of organic chemistry. During the slow writing of this book, which cost infinite pain and labour, Wade made himself a profoundly learned organic chemist. He went to original sources for every fact, and being gifted with a most retentive memory and an active imagination

he stored up an immense amount of knowledge. At the same time his mind was greatly stimulated by contact with the original writings of master chemists. His marked critical power and sense of proportion marshalled the facts of the science into an ordered edifice within his mind, and he became, as I have claimed, really learned in his subject. He produced an admirable book, but, what is more important, the writing of the book made its author an enthusiast[1].

Wade's career was made remarkable by the fact that he was, intellectually, entirely self-made. Few have owed so little to the help of others, or to education in the accepted sense of the word. The only educational scientific lectures he ever attended—save a few connected with evening classes at the Birkbeck Institute—were those he heard as an untaught lecture assistant, and so far as he listened to them at all it was as a youth not yet aware that chemistry was to remain his life's pursuit. He never, at any time of his life, so much as saw a foreign laboratory, and never once shared the responsibility of research work with a senior; never, indeed, saw any research in progress save that inspired by himself. Yet he made his influence deeply felt in the region of science which occupied him, and had he been spared but a few years longer I am convinced that his position among chemists would have been high indeed. He lost his life when his most productive period was beginning. His temperament, his craving for perfection in method and in accomplishment, made the rough adventures of research a trial to him. There were periods, indeed, in his earlier life when he seemed unable to face such adventures, and his output became small; but during the years which immediately preceded his death he acquired great momentum and was producing much. Work, indeed, became nothing short of an obsession with him, and he spared himself so little that those about him became anxious lest he should break down. The interruption to the work came, however, on unexpected and tragic lines, and was, alas, final.

In July, 1912, he was riding a motor cycle when he collided with a cart which had suddenly turned a corner of the road. A shaft struck him violently in the upper part of the chest, fracturing four ribs and damaging a lung. After surviving for three weeks and giving his friends good reason to hope for his ultimate recovery, he died suddenly on August 15th, 1912, as the result of pulmonary embolism.

As Wade's original work dealt with pure chemistry it would be out of

[1] Certain paragraphs in this notice are quoted from an account of Wade's life and work written by myself for the Chemical Society, *J. Chem. Soc.* **1913, 103, 767.**

place to discuss it here. I have given a moderately full account of it elsewhere[1]. One may point out, however, that in 1902 he published a paper on the constitution of the cyanides which takes classic rank; while from this time onwards he was continuously engaged in a series of extraordinarily careful investigations into the properties of mixtures with a constant boiling point. The choice of this line of research was no accidental matter. Wade was always attracted to the view that atomistic hypotheses are unnecessary to the foundations of chemistry, and believed that all the phenomena are capable of explanation on dynamic lines. He thought that such a dynamical basis would ultimately be found in facts foreshadowed in the properties of "hylotropic" mixtures. How the rehabilitation of the atom by modern physics might have finally affected his views I cannot say. He was always a bold, original and independent thinker.

Wade left a widow and one son. The latter, a youth of great promise, obtained last October an entrance scholarship at King's College, Cambridge. After this success he immediately applied for a commission, and is now (Feb. 1916), like other youths of promise, serving his country at the Front.

F. Gowland Hopkins.

CHARLES FREDERICK MYERS-WARD.

C. F. Myers-Ward was born in Birmingham in 1865, and was educated at the High School, New Street; he proceeded afterwards to Queen's College, and Mason College, where he received his medical education. After qualification in 1893 he practised in Birmingham for about two years, and then went to the Owens College, Manchester, as it was then known, to take up the position of Senior Demonstrator of Physiology. A few years later he was elected to the Professorship of Physiology at the University of Sheffield, and while holding that chair was invited to take the Lectureship on Physiology at Charing Cross Hospital Medical School; he accepted this invitation, and his business capacity was soon recognised there by his appointment as the Dean of that School. When Charing Cross a few years ago decided to give up the teaching of the Preliminary and Intermediate subjects of the curriculum, he followed the Charing Cross students to King's College, London, and received the title of Lecturer on

[1] *J. Chem. Soc.* 1913, **103**, 767.

Physiology at that Institution, where he worked successfully and wholeheartedly with Professor Halliburton. He was left in sole charge of the department when the latter left England for a prolonged holiday in 1914, but his occupancy of this position was only a short one, for to the universal regret of all his friends, colleagues and pupils, he died quite suddenly from heart-failure whilst staying at the seaside in August of that year. He married in 1894, Maud, younger daughter of Dr Morris of Birmingham, by whom he had two children, a son and a daughter, who with their mother survive him.

He published papers on "The Structure and Functions of the Mammalian Epididymis," "A Central Chronograph for Physiological Laboratories," and at the time of his death was engaged in a laborious research on Lipase; his results have been incorporated in a paper on that subject written by Dr Umeda and published in this *Journal*.

He devoted his time mainly to making his practical Histology classes as near perfection as possible, and left behind him a unique collection of histological material which King's College was fortunate enough to acquire. He devoted infinite pains to everything he undertook, and as a teacher he was second to none. He had an attractive personality, and not a little of his success was due to the respect and affection he inspired among those associated with him.

W. D. H.

CHARLES GLASS PLAYFAIR LAIDLAW.

C. G. P. Laidlaw was born in 1887 in London but he was of pure Scotch descent. He was educated at the Perse School, Cambridge, and at St John's College, Cambridge. In 1912 he obtained a Board of Agriculture Research Scholarship in Plant Physiology. This scholarship he held at the Imperial College of Science and Technology. In the autumn of 1914 he joined the London Scottish regiment. He left England for the front in March, 1915. He was wounded early in April in the trenches at Richebourg l'Avouée and died shortly afterwards; a very promising career was thus cut short. Laidlaw was a very recent member of the Society which he joined only in 1913. The only publication for which he was responsible was one in plant physiology (in collaboration with Mr R. C. Knight) dealing with a new type of recording apparatus for investigating the size of stomata.

V. H. B.

CHARLES MARTIN.

It is with deep regret that we have to record the death of Charles Martin of Abergavenny who was killed in action on May 3rd, 1915, near Ypres.

Martin was educated at Eton. He had not, however, come out in his early days, and he appeared to his contemporaries only as a "modest, simple-minded, rather shy boy, and without the school accomplishments which make boy heroes." He found the lessons boring and got few good reports. Later on he went to Oxford and entered Magdalen College, the President of which was his mother's cousin, and, being a keen field naturalist, he selected Zoology as the subject most likely to interest him. After taking his degree he spent a summer with the Survey of the Freshwater Lochs of Scotland, and published observations on certain Oligochaets and Turbellaria found there. Subsequently he proceeded to the Zoological Station at Naples, and on his return made a brief stay at the Eustace Gurney Laboratory in Norfolk; he was then appointed Demonstrator of Zoology in the University of Glasgow. This post was relinquished in 1911, when he came south, and worked partly at Rothamsted, partly at the Lister Institute, and partly in his own laboratory at his home in Wales.

From the Turbellaria he passed to the Acinetaria, and then to the Protozoa on which most of his work was done. Most of his Papers were published in the *Quarterly Journal of Microscopic Science*; his last two, however, were in the *Philosophical Transactions* and in *The Journal of Agricultural Science* —both on Soil Protozoa.

The great characteristic of Martin's work was the freshness of his outlook. He never would be bound by tradition or convention, or even by time or circumstance. His work, therefore, was really original; he looked at the problem from a new point of view, attacked it in a new way, and always got an interesting result. This was well shown in his work at Rothamsted. He was at once attracted by the hypothesis that soil protozoa constitute one of the factors keeping down the bacterial numbers in the soil, and with characteristic enthusiasm he speedily struck a way through the experimental difficulties with which the subject bristled. He devised a method which for the first time demonstrated the presence of trophic amoebae in the soil and isolated and studied typical organisms present. His naturalist's instincts led him to make some very shrewd faunistic observations, which are being fully confirmed by later work.

A mere discussion of Martin's work, interesting and valuable as it would be, is wholly inadequate to give any picture of what he was like in the Laboratory. In Martin's case you cannot separate the work from the man. While he was with us it was difficult to speak of him without affection, and now that he has gone it is impossible to think of him without emotion. Always boisterously cheerful, never serious, and yet always serious—for he would break off in the midst of the wildest chaff to plunge into the most truculent piece of laboratory work—always uncertain in the minor affairs of life, he was yet firm as a rock in all the things that really matter. You never knew when he would come, how long he would stay, or where he would go next, but you could always know that whether he was here or there he was always the same straight, honest man, free from any taint of unkindness or uncharitableness. And, whether his stay in the Laboratory was long or short, he would bring in a refreshing breeze from the open, and leave behind a wealth of illuminating suggestions.

He was that essentially British product, an amateur in science; a man possessed of private means, a good country house, considerable personal charm and social attainments, moving in a wide circle, and leading a great open life, who from among many possible paths chose that of science because he loved it. So long as we have such men British science can never be without the joy of the enthusiast, and can never degenerate into a mere trade. But just as he refused to be tied down by convention, so he refused to be tied down by science. At Oxford he had run the Magdalen beagles, and at home he was Master of the Crickhowell Harriers, besides fulfilling the other functions of the country gentleman. He had joined the O.T.C. at Glasgow, and when the war broke out went with the Monmouths. It was in the great battle early in May that he was killed by shrapnel while in charge of the machine-gun section. We may be sure that he gave a good account of himself, and that he faced death as fearlessly as he had faced life. It was thus that he would have preferred to go; striking hard and doing something big: dying in defence of those ideals of honour amidst which he had always lived.

E. J. Russell.

ARTHUR CECIL HAMEL ROTHERA.

ARTHUR CECIL HAMEL ROTHERA was born on July 1st, 1880, at Nottingham. After receiving a sound education at Market Bosworth Grammar School he entered Emmanuel College, Cambridge. I was in close touch with him throughout his University career and from first to last was impressed with his sterling worth and with the high standards of conduct and endeavour which he kept always before him. Even as a youngster fresh from school he took a serious view of his responsibilities: a view indeed which, at the time, was perhaps too serious to be fully understood by his contemporaries. He won friends, however, of the best type, who remained faithful to him and will be faithful to his memory. Hard work, coupled with abilities which were above the average, assured his success at Cambridge. He obtained a first class in the Natural Science Tripos Part 1, in June, 1902, and in the following year a first class in Part 2, his subject being physiology.

He was early attracted towards the chemical side of physiological studies, and after obtaining his degree he came to tell me that he would look to biochemistry for a career. As a result of this decision, which at that time called for some courage, he determined to obtain a medical qualification as quickly as possible and then return to Cambridge for research in his chosen subject. He went to St Mary's Hospital and in 1904, after having performed the duties of some of the essential clinical appointments, but, as yet, unqualified, he came back to the University and spent some months upon a study of cystine and sulphur metabolism. During the same year he went to Strassburg and carried out a brief research in the domain of protein chemistry under Hofmeister. Later he returned to hospital for further medical work while occasionally visiting Cambridge to obtain laboratory experience.

In the year 1906, as the result of the influence of Professor W. A. Osborne, a lectureship in biochemistry was created in the University of Melbourne.

As a member of a small committee which was asked to select candidates for this post I felt justified in pressing Rothera's claims and he was eventually appointed to it. The opportunity came rather early in his career. He was young, somewhat shy and reserved, and he possessed, of course, no great experience either of teaching or research. To place him in so important a post, one of the very first to be definitely associated with the name of the

academically new subject of biochemistry, was certainly of the nature of an experiment. It is therefore a source of keen pleasure to those who had faith in him to read the words of Professor Osborne, his senior colleague at Melbourne, written after Rothera's nine years of service and untimely death.

"Dr Rothera," writes Professor Osborne, "was a biochemist of the best type....With all those varying activities with which biochemistry comes into contact he was in the closest sympathy. The medical student had the value of his teaching in the physiology class room, and in the lectures on pharmacology, but he had little opportunity of realising the splendid service which was rendered to agriculture, not only in the agricultural biochemistry course which Dr Rothera created here, and which inspired every pupil who took up the subject, but in special research work for the Agricultural Department. To-day those who have the future of scientific agriculture at heart are deploring with us the loss of a highly skilled adviser and investigator who we fear cannot easily be replaced." Professor Osborne says further, "Any estimate of Dr Rothera's life work which was restricted to his publications and purely professional duties would be very inadequate. For he influenced pupils and friends by the charm of his personality, by the integrity of his character and by the inexhaustible loyalty of his friendship." This is high praise from one who was in close touch with the man he is describing.

I am tempted to recall the circumstances of Rothera's preparation for his first journey to Melbourne as they illustrate his grit. The appointment came somewhat unexpectedly, and he was left with a very brief period—I believe it was a fortnight only—to wind up his affairs in this country, to get his equipment together, to sit for the final examination of the Conjoint Board, and, above all, to cross the Channel and persuade the family of the charming French lady who was to be his wife to consent to an arrangement which would later on involve her joining her husband in a country most remote. All was successfully accomplished in the brief period mentioned and Rothera sailed to begin a career which though it was to prove lamentably short was to bring him satisfaction and happiness.

After the war began I received letters from him which showed unrest of mind. He was unable to settle to any work which did not involve direct service to his country in her need. In August, 1915, he broke away and with the consent of the University he obtained a Captaincy in the R.A.M.C.: but the service he longed to give was unhappily soon terminated. He contracted influenza while working in camp, and a fatal attack of pneumonia led to his death on Sunday, Oct. 3rd, 1915. He died with his scientific work

little more than begun, but the subject of his choice has benefited from his labours and his colleagues and successors will not forget what he accomplished.

The circumstances of his death were certainly most sad and lent more than ordinary pathos to the loss suffered by his widow and her two young children. Yet though his career was short and his end untimely it must not be forgotten that his work had always been such as he loved and enjoyed.

In 1905 Rothera published a paper dealing with a modification of the nitroprusside test for acetone which has proved extremely useful. As Professor Osborne remarks, one can say without exaggeration that it is used in practically every medical school and hospital throughout the world. Rothera soon realised, when he found himself at work in Melbourne, that in a new country the University teacher must give attention to practical problems. Much of his time and energy were expended upon a study of the chemistry and physiology of milk. These studies bore upon the needs of local industries but they also possessed scientific value. Some of this work was published, but much of it may now never see the light of publication. His last paper (with Mr L. A. L. Maxwell) described experimental results, which must have been very difficult to obtain, in proof of his belief that the action of pituitary extracts in promoting the flow of milk is the result of a true secretory stimulus and not merely due to muscular contractions.

F. Gowland Hopkins.

I. THE FATE OF INORGANIC NITROGEN IN THE METABOLISM OF THE DOG.

By WILLIAM CALDWELL
and HAROLD REX SEPTIMUS CLOTWORTHY.

(*Received December 11th, 1915.*)

These experiments were begun in January, 1913, and were undertaken with the object of confirming or otherwise the generally accepted view which prevailed at that time, namely, that retention of nitrogen takes place when the proteins of the food are replaced by inorganic nitrogen in the form of ammonium salts. As the investigation—the results of which are contained in Table I—proceeded, it was thought that it would be an interesting addition to the work to see in what forms the nitrogen was excreted, and if it was retained, to determine whether the retention was temporary or permanent.

The meaning of the expression "retention of nitrogen" is somewhat ambiguous. If the weight of the animal remains constant or increases, and some or all of the extra nitrogen given in the food or injected into the body is retained at the same time, then undoubtedly nitrogen is retained. On the other hand, if more nitrogen is excreted than is given with the food or injected into the body, although the weight of the animal increases, it is assumed that no nitrogen is retained. Again, if the weight of the animal decreases, and some of the extra nitrogen given is not immediately excreted, it is assumed that the decrease is due to a loss of water, and that in this case there is retention of nitrogen, though it may be temporary.

Peschek [1911] was the first to call attention to this problem. He fed an animal on food which contained a high percentage of nitrogen, and added ammonium acetate for certain periods to the food. His results, however, were contradictory—sometimes nitrogen was retained, and at other times it was lost.

The problem has since been investigated by a number of authors. Grafe and Schlaepfer [1912] assert that an animal has the power to retain nitrogen and use it to synthesise protein from nitrogen-free food (carbohydrates and fats) and inorganic nitrogen in the form of ammonium salts. Abderhalden [1912] also came to the conclusion that clear evidence was obtained of the formation of protein, when dogs were fed on a nitrogen-free food with the addition of inorganic nitrogen. But on an examination of his results it will be seen that in the first experiment there is a negative nitrogen balance and a continual loss in weight. Similarly in Exp. 2 there is a negative nitrogen balance and a continual loss in weight. In Exp. 3 a dog which had lost 2000 g. recovered its original weight rapidly—in 11 days—by feeding with the hydrolysed products obtained from flesh and blood. The dog was then fed on a nitrogen-free diet, and the animal lost weight, and the nitrogen balance was negative during this part of the experiment. Then ammonium acetate was added to the carbohydrate and fat food. The nitrogen balance was positive for five days and negative for the remainder of the period. The result of the feeding for the whole period was a retention of nitrogen and a slow but gradual decline in weight. In the last period of this experiment the amount of ammonium acetate given with the carbohydrates and fats was increased but the nitrogen balance remained negative during the whole period—5·34 g. nitrogen being lost—and the weight fell gradually but slowly. In Exp. 4 the nitrogen balance was negative during the whole of the experiment. Thus what Abderhalden has proved, apart from confirming the established fact, that the protein of the food may be replaced by the completely hydrolysed products obtained from flesh and blood, is, that the addition of inorganic nitrogen to a nitrogen-free food prevents the rapid loss of weight which takes place when an animal is fed on carbohydrates and fats alone. His experiments do not conclusively show a permanent retention of inorganic nitrogen and Abderhalden has lately admitted this. At the end of a long investigation [Abderhalden, 1913], he states that ammonium salts, and especially ammonium acetate, can influence at times the nitrogen balance in the sense of a retention, but, he adds, there are no grounds, from the results which he has obtained, for the assumption that protein can be formed from ammonia and nitrogen-free compounds, such as carbohydrates and fats. More recently the problem of the retention of nitrogen has been investigated by Taylor and Ringer [1913] who find that the presence of carbohydrates in the food is not a necessary factor in the retention of nitrogen from ammonium salts; and also by Underhill [1913, 1], whose communications are

important. He fed a dog on an adequate mixed diet. The ammonium salts of organic acids were given to the dog orally, and it was found that none of the added nitrogen was excreted as ammonia nitrogen. When however the ammonium salts of inorganic acids were given to the dog, the added nitrogen was excreted partly as ammonia nitrogen—with ammonium chloride 52 % of the added nitrogen, and with ammonium phosphate 64 % of the added nitrogen appeared in the urine. In a later paper Underhill [1913, 2] contests the conclusions of Grafe and Schlaepfer whose work he repeats but fails to find evidence of any of the inorganic nitrogen being used in the synthesis of proteins.

The analogy between the plant cell and the animal cell does not seem to hold in respect to nitrogen; for, while syntheses are not denied to the animal cell, it has not yet been shown that it is able to synthesise complex organic substances from inorganic constituents. Again, we are much inclined to discuss results as if an animal were a perfect mechanical balance, which it is not; for, whilst approximate nitrogenous equilibrium can be attained, absolute nitrogenous equilibrium can scarcely be said to exist. A fair margin has to be allowed for temporary fluctuations on either side of the line of absolute equilibrium.

Method.

We fed a dog on a fixed diet containing a definite amount of nitrogen as determined by analysis, for a considerable time before the actual experiment began, to enable the dog to acquire a steady state or condition in which the amounts of nitrogen excreted per day should be approximately equal. When this steady state had been attained a known amount of an ammonium salt was added to the food—one-half to the food in the morning, and the remaining half to the food in the evening. This was continued for three or four days. An interval of three or four days was then allowed to elapse before a second ammonium salt was added to the food, and so on. Our feeding periods therefore extended for three or four days and the intervals after the addition of the extra nitrogen also extended for three or four days. The bladder was emptied regularly by a sterilised catheter at intervals of 24 hours. The day's urine was measured and a complete analysis was performed each day, whether during a period or an interval. The faeces were collected and grouped into periods corresponding to the experimental and the interval periods.

The amount of water which the dog drank was accurately recorded.

In some of the experiments the ammonium salt was injected subcutaneously in sterile condition—half of the volume was injected in the morning after food, and the other half injected in the evening after food.

These injections were made for us by Professor W. H. Thompson.

The diet consisted of 100 g. of potatoes, 100 g. dog biscuit, 50 g. flour, 10 g. dried milk, 10 g. lard, 2 g. salt and 250 g. water.

Dog XV.

The results obtained with dog XV are shown in Table I.

The investigation of the results of feeding with ammonium salts had been in progress for some time before analyses to ascertain the manner in which the extra nitrogen was excreted were undertaken. As a consequence the details of the early part of the experiment are in this respect not complete.

Period III. 0·617 g. nitrogen was added to the food as ammonium chloride and 0·939 g. appeared—0·312 g. or 33·2 % as ammonia nitrogen and 0·458 g. in the faeces.

Though there was a positive balance, yet compared with period II there was a loss of nitrogen.

Period IV. During this interval the amount of ammonia nitrogen decreased and became almost equal to the amount of ammonia nitrogen during period II. The period was a long one and may be compared with the succeeding feeding period.

Period V. 0·413 g. nitrogen was added to the food as ammonium carbonate. It will be seen that the amount of ammonia nitrogen has not been increased by the addition of ammonium carbonate to the food.

Period VI. The amount of ammonia nitrogen remained approximately steady.

Period VII. 0·315 g. nitrogen was added to the food as ammonium phosphate; more than this amount of nitrogen appeared, as may be seen on comparing the results with period VI or with period II. The amount of ammonia nitrogen was increased by 0·331 g.—an amount greater than the amount of nitrogen added.

Period VIII. During this interval the amount of ammonia nitrogen again became approximately normal, but on the addition of sodium phosphate to the food, the amount of ammonia nitrogen excreted was decreased, and the amount of urea nitrogen increased.

From this investigation it would appear therefore that the amount of ammonia nitrogen excreted depends on the nature of the ammonium salt

TABLE I. Dog XV.

Period	Weight g.	Nitrogen given per day g.	Nitrogen excreted per day g.	Nitrogen balance g.	Nitrogen added g.	Nitrogen excreted g.	Urea nitrogen g.	Ammonia nitrogen g.	Creatinine mg.	Creatine mg.	Nitrogen in faeces g.
I. 6 days. Ammonium acetate (in food)	7,003	4·369	3·786	0·583 +	0·539	—	—	0·0726	192·9	44·7	0·942
II. 8 days. Interval ...	7,048	3·830	2·844	0·986 +	—	—	—	0·0749	199·8	43·7	0·317
III. 6 days. Ammonium chloride (in food)	7,156	4·447	3·783	0·664 +	0·617	0·939	—	0·3869	207·8	24·8	0·775
IV. 8 days. Interval ...	7,282	3·830	3·163	0·667 +	—	0·319	—	0·0847	208·0	26·8	0·634
V. 6 days. Ammonium carbonate (in food)	7,424	4·243	3·542	0·701 +	0·413	0·698 (0·379)	—	0·0737	197·1	25·3	0·713
VI. 3 days. Interval ...	7,535	3·830	3·562	0·268 +	—	0·718	—	0·0676	195·1	20·3	0·697
VII. 6 days. Ammonium phosphate (in food)	7,609	4·145	4·040	0·105 +	0·315	1·196 (0·478)	2·3599	0·3981	204·5	45·3	0·731
VIII. 3 days. Interval	7,779	3·830	3·490	0·340 +	—	0·646	1·756	0·0733	199·4	20·7	0·786
IX. 5 days. Sodium phosphate (in food)	7,824	3·830	3·620	0·210 +	—	0·776	2·153	0·0366	208·6	25·1	0·855
X. 3 days Interval ...	7,929	3·830	3·524	0·306 +	—	0·680	1·977	0·0550	197·3	26·6	0·752

added to the food. The ions into which the ammonium salts are dissociated may or may not act as foreign substances in the body. In the case of ammonium carbonate, the body does not find it necessary to protect itself but in the cases of ammonium chloride and ammonium phosphate it would seem that the decomposition of the ammonium salt demands from the body some kind of protection from the hydrochloric acid or the phosphoric acid, and the ammonia produced is excreted; hence the increase in the amount of excreted ammonia nitrogen.

The addition of sodium phosphate to the food is somewhat interesting. Di-sodium hydrogen phosphate is slightly hydrolysed in solution and has an alkaline reaction. The effect of such a substance would seem to decrease the amount of ammonia nitrogen excreted and increase the amount of urea nitrogen excreted.

The conclusion we may legitimately draw from the foregoing investigation is that an ammonium salt of a weak acid has little or no effect on the amount of ammonia nitrogen excreted; whereas the ammonium salt of a strong acid increases very considerably the amount of excreted ammonia nitrogen.

While the nitrogen balance was positive during the whole experiment, the amount of nitrogen excreted increased during the course of the experiment. There was therefore no retention of the added nitrogen during this series of experiments.

Dog XVII. (Table II.)

Dogs XV and XVIII were reserved exclusively for the investigation of the metabolism of the ammonium salts mentioned in the tables which record the results. These dogs were kept for a very considerable time on a constant diet before the real investigation began. Dog XVII was not treated in such a systematic manner.

In this investigation the ammonium salts of lactic acid and citric acid were used. When the salts were injected there was not a very marked increase in the amount of the ammonia nitrogen excreted. The amount of urea nitrogen excreted increased when ammonium lactate was injected and decreased when ammonium citrate was injected. The behaviour of these salts on injection, however, is in strange contrast to the behaviour of the same salts when they are added to the food.

During period IV, 0·550 g. nitrogen was added to the food as ammonium lactate, and 0·480 g. nitrogen appeared as excreted nitrogen. Of this excreted nitrogen 0·436 g. or 90·8 % appeared as urea nitrogen. During period VI

TABLE II. Dog XVII.

Period	Weight g.	Nitrogen given per day g.	Nitrogen excreted per day g.	Nitrogen balance g.	Nitrogen added g.	Nitrogen excreted g.	Urea nitrogen g.	Ammonia nitrogen g.	Creatinine mg.	Creatine mg.	Nitrogen in faeces g.
I. 5 days. Interval ...	8,208	3·83	3·908	0·078 –	—	—	2·615	0·155	261	12	0·875
II. 5 days. Ammonium lactate (injection)	8,293	4·09	4·326	0·236 –	0·260	0·418	2·707	0·178	264	20	0·9298
III. 4 days. Interval ...	8,341	3·83	3·504	0·326 +	—	—	2·371	0·163	233	41	0·643
IV. 5 days. Ammonium lactate (in food)	8,372	4·38	3·984	0·396 +	0·550	0·480	2·807	0·148	240	58	0·656
V. 3 days. Interval ...	8,419	3·83	3·905	0·075 –	—	—	2·465	0·148	232	55	0·920
VI. 5 days. Ammonium citrate (in food)	8,494	4·38	4·283	0·097 +	0·550	0·378	2·741	0·144	234	49	0·833
VII. 3 days. Interval ...	8,548	3·83	3·660	0·170 +	—	—	2·381	0·132	239	49	0·777
VIII. 3 days. Ammonium citrate (injection)	8,561	4·10	3·996	0·104 +	0·270	0·336	2·308	0·168	242	51	0·931
IX. 3 days. Interval ...	8,590	3·83	3·685	0·145 +	—	—	2·393	0·146	238	53	0·816

0·378 g. nitrogen appeared as excreted nitrogen after the addition of 0·550 g. nitrogen to the food as ammonium citrate, and of this excreted nitrogen 0·276 g. or 73 % appeared as urea nitrogen.

Dog XVIII. (Table III.)

Period I. The steady state is represented by:—3·830 g. nitrogen given per day, and 3·545 g. nitrogen excreted per day.

Period II. 0·47 g. nitrogen was added to the food as ammonium acetate, and of this 0·424 g. appeared as excreted nitrogen; 0·383 g. or 90·3 % of this excreted nitrogen was in the form of urea nitrogen.

0·046 g. nitrogen was retained during the period, but this amount was lost in the succeeding interval. Not only so, but a loss of 0·914 g. nitrogen occurred in interval III and this nitrogen was excreted in the faeces of interval III. The added nitrogen therefore seems not only to be excreted, but to have caused some decomposition of nitrogenous substances. The result of feeding with ammonium acetate is therefore an unaccountable loss of nitrogen. It may be a washing out of temporarily retained nitrogen.

There was a decrease of 100 g. in the weight of the dog during the interval III.

Period IV. 0·283 g. nitrogen was injected as ammonium acetate, and 0·675 g. nitrogen appeared as excreted nitrogen. 0·443 g. or 60·6 % of this excreted nitrogen appeared as urea nitrogen, and 0·12 g. nitrogen appeared as excess in the faeces. There was thus a loss of nitrogen during the period.

Period V. During this interval there was a very slight loss of nitrogen compared with the state of equilibrium which existed at the beginning of the experiment.

Period VI. 0·61 g. nitrogen was added to the food as ammonium chloride and of this 0·490 g. appeared as excreted nitrogen. In comparison with the "steady state" at the beginning of the experiment, 0·297 g. or 60·5 % was excreted as urea nitrogen, and 0·327 g. or 66·7 % as ammonia nitrogen. But in comparison with interval V, 0·135 g. or 27·5 % appeared as urea nitrogen, and 0·354 g. or 72·2 % as ammonia nitrogen.

0·12 g. nitrogen was retained at the end of the period.

Period VII. During this interval not only was the retained nitrogen lost, but 0·416 g. extra nitrogen was excreted, principally in the faeces. The urea nitrogen excreted was 0·202 g. less than normal, whilst the nitrogen in the faeces was 0·67 g. more than in the normal condition. Most of this extra excreted nitrogen appeared on the day following the previous period.

TABLE III. Dog XVIII.

Period	Weight g.	Nitrogen given per day g.	Nitrogen excreted per day g.	Nitrogen balance g.	Nitrogen added g.	Nitrogen excreted g.	Urea nitrogen g.	Ammonia nitrogen g.	Creatinine mg.	Creatine mg.	Nitrogen excreted in faeces g.
I. 5 days. Interval ...	6,735	3·830	3·545	0·285 +	—	—	2·2861	0·1919	176·76	68·02	0·790
II. 5 days. Ammonium acetate (in food)	6,887	4·300	3·969	0·331 +	0·47	0·424	2·6692	0·1878	175·62	96·10	0·7878
III. 4 days. Interval ...	6,787	3·830	4·459	0·629 –	—	0·914	2·2673	0·1857	176·85	38·15	1·741
IV. 3 days. Ammonium acetate (injected) ...	6,837	4·113	4·220	0·107 –	0·283	0·675	2·7292	0·1608	182·6	21·3	0·912
V. 3 days. Interval ...	6,771	3·830	3·576	0·254 +	—	0·031	2·4482	0·1658	170·6	26·4	0·9037
VI. 5 days. Ammonium chloride (in food)	6,830	4·440	4·035	0·405 +	0·61	0·490	2·5834	0·5190	181·2	32·1	0·825
VII. 3 days. Interval ...	7,016	3·830	3·961	0·131 –	—	0·416	2·0849	0·2081	177·8	21·0	1·461
VIII. 5 days. Ammonium chloride (injected)	6,980	4·418	4·217	0·201 +	0·588	0·672	2·4746	0·4150	189·8	27·7	0·855
IX. 2 days. Interval ...	6,938	3·830	3·075	0·755 +	—	0·470 (Retained)	2·3040	0·1925	184·7	78·8	0·3495
X. 3 days. Ammonium phosphate (in food)	7,107	4·281	4·210	0·071 +	0·4508	0·665	2·2941	0·3989	191·6	32·0	1·267
XI. 2 days. Interval ...	7,122	3·830	3·635	0·195 +	—	0·090	2·0741	0·2429	192·7	27·1	1·063
XII. 3 days. Ammonium phosphate (injected)	7,268	4·334	3·680	0·654 +	0·504	0·135	2·4377	0·3103	203·4	40·3	0·584
XIII. 2 days. Interval ...	—	3·830	—	—	—	—	2·0594	0·3136	179·3	71·9	—

Period VIII. 0·588 g. nitrogen was injected as ammonium chloride, and 0·672 g. nitrogen appeared as excreted nitrogen. In comparison with the "steady state" 0·1885 g. or 28 % appeared as urea nitrogen, and 0·2231 g. or 33·2 % as ammonia nitrogen; whereas in comparison with the previous period 0·3897 g. or 58 % appeared as urea nitrogen, and 0·2069 g. as ammonia nitrogen—an amount very slightly in excess of the added nitrogen injected.

Period IX. During this interval a comparatively large amount of nitrogen was retained from the regular food, 0·755 g. in comparison with 0·285 g. during the equilibrium stage at the beginning of the experiment. The amount of urea nitrogen became normal, and so also did the ammonia nitrogen, whilst the amount of nitrogen excreted in the faeces was 0·45 g. less than during the first period.

Period X. 0·4508 g. nitrogen was added to the food as ammonium phosphate, and 0·665 g. appeared as excreted nitrogen. The urea nitrogen excreted remained normal, but the ammonia nitrogen was 0·207 g. (or 31 % of the excreted nitrogen) in excess of that of the equilibrium stage. 0·47 g. nitrogen was excreted in the faeces more than the normal amount. This amount and the amount of extra urea nitrogen excreted make up the total amount excreted.

Period XI. During this interval some nitrogen was lost—0·09 g. The urea nitrogen excreted was 0·22 g. less than in the normal state, but against that the faeces contained 0·27 g. nitrogen in excess of that of the first period.

Period XII. 0·504 g. nitrogen was injected as ammonium phosphate and 0·135 g. appeared as excreted nitrogen. 0·15 g. appeared as urea nitrogen, and 0·12 g. as ammonia nitrogen, whilst 0·21 g. nitrogen less than the normal amount was excreted in the faeces. In this period a very considerable amount of nitrogen is retained.

It was noted during the experiment that the amount of ammonia nitrogen varied directly as the acidity of the urine, determined by titration with N/10 alkali, using phenolphthalein as indicator. The urine itself, as was to be expected, was never alkaline to phenolphthalein. The exact determinations will be published in a later paper.

An interesting point is to compare the periods of feeding with the periods of injection in the foregoing experiments.

1. The experiments with ammonium chloride (Table III, periods V–VIII). On comparing the interval immediately before the feeding experiment with the feeding period itself, it will be seen that the extra nitrogen which was excreted appeared as 0·135 g. or 27·5 % urea nitrogen, and 0·354 g.

or 72·2 % ammonia nitrogen, whereas in the case of the injection of ammonium chloride, the extra nitrogen which was excreted appeared as 0·3897 g. or 58 % urea nitrogen, and 0·2069 g. or 30·8 % ammonia nitrogen.

The injection seems to reverse the magnitude of the amounts of excreted nitrogen as urea nitrogen and as ammonia nitrogen respectively, when compared with the feeding experiment.

2. The experiments with ammonium phosphate (Table III, periods IX–XIII). On comparing the interval immediately preceding the feeding experiment with the feeding experiment period, it will be noticed that the extra excreted nitrogen appears as ammonia nitrogen only, but in the case of the injected ammonium phosphate, the extra excreted nitrogen appears as 0·36 g. urea nitrogen and 0·07 g. ammonia nitrogen, a reversal of magnitude similar to the experiments with ammonium chloride.

3. The experiments with ammonium acetate (Table III, periods I–IV). On comparing the feeding experiment with the interval immediately preceding the feeding experiment, it will be seen that the extra excreted nitrogen appears as urea nitrogen only. The ammonia nitrogen is almost identical with that of the previous period. Similarly with the injected ammonium acetate. The extra excreted nitrogen appears as urea nitrogen only, but the ammonia nitrogen is 0·025 g. less than the previous period.

There is no such marked reversal as that noted in the cases of ammonium chloride and ammonium phosphate.

Taking the experiment as a whole there was no retention of the added nitrogen. The animal was excreting more nitrogen at the end of the experiment than during the "steady state" period; yet the weight of the animal had increased by more than 500 g. during the investigation, which lasted about nine weeks.

The health of the animal during the experiment was excellent.

Discussion and Summary.

Speculations concerning the synthetic powers of the body under conditions such as we have been investigating are very attractive, but we hesitate to make positive assertions. Even when the extra nitrogen is given in the form of ammonium salts, and it appears as urea, we hesitate to say that the ammonia nitrogen is synthesised into urea. It is no doubt true that the ammonium salt is dissociated. That is evident when we consider the feeding experiments with ammonium chloride. The dissociation of the ammonium chloride into ammonia and hydrochloric acid and the separation of these

products involve a kind of protection on the part of the body, and the formation of a large amount of ammonia, which is subsequently excreted, to protect itself against the acidity of the hydrochloric acid. If the urea is produced from ammonia and carbon dioxide it will involve not only the dissociation of the ammonium chloride, but the oxidation of the ammonia and the reduction of the carbon dioxide in a fashion which we shall not discuss. We do not suggest that the nitrogen is not made use of in synthesis or replacement. It may be, though, that the ammonium salts have some other action, such as displacement, with decomposition of the substances which produce urea.

The chief interest in our work is the manner in which the extra nitrogen taken with the food or injected into the body is excreted. The form in which it is excreted depends on the ammonium salt employed, and apparently on the ions into which the ammonium salt is dissociated. For instance, the added nitrogen in the case of ammonium acetate is excreted in the form of urea. In the case of ammonium chloride and ammonium phosphate, it is excreted both as urea and ammonia. The acetic acid derived from ammonium acetate is a much weaker acid than the hydrochloric acid, or the phosphoric acid derived from the ammonium chloride and ammonium phosphate respectively. The body does not find it necessary to protect itself by the production of ammonia, to neutralise the acetic acid, but it is compelled to protect itself in the case of hydrochloric acid and phosphoric acid.

The form in which the added nitrogen is excreted depends also on whether the nitrogen is taken with the food, or injected into the body. When the ammonium salt is injected the output of urea is greatly increased, and the amount of nitrogen excreted in the faeces is much less than in the previous periods.

Professor W. H. Thompson very kindly performed the injections for us, and we are sincerely indebted to him for his valuable suggestions and advice during the course of the investigation.

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II. ON THE DEVELOPMENT OF THE BLACK MARKINGS ON THE WINGS OF *PIERIS BRASSICAE*.

By HERBERT ONSLOW.

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The formation of black animal pigments or melanins has been shown by Biedermann [1898], Gortner [1910, 1911, 1] and other investigators to be due to the oxidising action of an enzyme upon a colourless chromogen. The chromogen is probably tyrosine or a closely allied substance and the enzyme a tyrosinase.

In order to investigate the factors which determine the formation of pattern, and if possible to extend our knowledge of the mechanism of melanin production, a study was made of the black wing-markings of the common large white cabbage butterfly (*Pieris brassicae*). Hopkins [1895] has already shown that the chalky white pigment deposited in the scales of these insects gives the murexide test and is in fact uric acid. He has also shown that the yellow pigment which occurs on the under-sides of the wings of *Pieris brassicae*, and which constitutes the sole pigment of the wings of *Gonepteryx rhamni*, gives the murexide test and is a derivative—probably an oxidation product—of uric acid.

The development of the black wing-markings of *P. brassicae*, which differ slightly in the male and female, has been observed by Urech [1892], but his account is chiefly descriptive and does not deal with the cause or chemistry of their production.

Von Fürth [1904] and Roques [1909] have shown that the haemolymph of certain pupae is rich in tyrosinase, and the former has described a tyrosinase obtained from the pupae of *Deilephila elpenor* similar in most respects to that mentioned in this paper.

For the material of the present study a number of pupae of *Pieris*

brassicae were obtained. These have a greyish green colour which remains until a short time before the emergence of the complete insect in May. A few days before this takes place the white wing-pigment makes its appearance through the chitin of the pupa-case, and a little later pale spots can be observed on the wing. If the pupa is dissected at this stage the markings do not appear black but yellowish, like spots of grease upon white paper. These markings begin to darken quickly and just before emergence become deep black. During this transition the chitinous case of the pupa becomes definitely separated from the body of the insect, so that the latter can be dimly seen lying within its semi-transparent sheath. It is probably during this period that sufficient air finds access to the wing to cause the oxidation in the black areas to take place. When fully developed this black pigment is deposited in minute granules within the chitin of the wing scales and is similar in appearance and solubility to the melanin of hair [Onslow, 1915] and other structures. It can be partially extracted by prolonged boiling in dilute alkali. It is soluble in weak alkalies, somewhat less soluble in weak acids (N/20 HCl) and insoluble in strong acids and saturated ammonium sulphate solution.

Experimental.

Several pupae were chloroformed just before the wing-markings began to blacken, and the haemolymph was extracted by grinding them in an agate mortar with a little kieselguhr and chloroform water. After filtration, the pale green slightly opalescent fluid obtained was made faintly alkaline to litmus with very dilute sodium carbonate solution and tubes were prepared each containing 2 cc. of the fluid. The following substances were then added to the tubes which were plugged with cotton-wool and kept at room temperature for a period of 12 hours.

Experiment I.

No.	Contents	Appearance after 12 hours
1	Haemolymph	Deep black at the surface
2	„ + tyrosine	„ „ „
3	„ + p-cresol	Blood-red
4	„ (boiled)	Unchanged

Since a deep black ring was formed at the surface of the fluid without the addition of tyrosine it is clear that the haemolymph was not only rich in tyrosinase but also in chromogen. It was found absolutely necessary to

keep the tubes at a temperature of 20° or less, for at higher temperatures formation of the pigment was inhibited. This observation is in agreement with that of von Fürth [1904] who found that a temperature of 30° completely inhibited the reaction, whereas tubes kept in the ice-chest were not affected.

It is interesting to note that in vertebrates the action of the tyrosinase is accelerated by a temperature of 36°. This difference might be accounted for by assuming that the organic peroxide constituent of the oxidase of insects is completely destroyed at a temperature of 30°. Unfortunately the author has not yet been able to test this hypothesis.

In order to ascertain whether any chromogen was present in the scales, the fore-wings were dissected from a number of pupae. The members of each pair of wings are distinguished in the following experiment by the letters *A* and *B*, *B* always serving as a control. The bodies of these pupae were utilised for the preparation of the haemolymph in the manner already described. Each wing was placed in a watch-glass containing the following solutions in which they remained for 12 hours at room temperature.

Experiment II.

No. of wing	Contents of watch-glass	Appearance after 12 hours
Pair 1 A_1	Normal saline	+
Pair 1 B_1 (boiled)	,,	−
Pair 2 A_2	Haemolymph	+ +
Pair 2 B_2 (boiled)	,,	+ +
Pair 3 A_3 ,,	,,	+ +
Pair 3 B_3 ,,	,, (boiled)	−
Pair 4 A_4	Saturated tyrosine solution	+ + +
Pair 4 B_4 (boiled)	,, ,, ,,	−

\+ indicates slight darkening of the markings.
\+ + ,, considerable darkening of the markings.
\+ + + ,, blackening of the entire wing.
− ,, no change.

It appears from this experiment that both the wing and the haemolymph carry the two factors necessary for melanin production, since it is only when both the wing and the haemolymph are boiled that no darkening takes place (B_3). It is probable that the haemolymph which contains both chromogen and ferment adheres to the surface of the wing and may even be present in the wing nervures. When the tyrosinase of the body lymph comes into contact with the chromogen present in the wing-markings it oxidises this more than it does the rest of the wing. A fourth pair of wings was prepared,

in order to determine whether the localisation which causes the formation of pattern is due to

(1) the restriction of the tyrosinase to the markings and the distribution of the chromogen over the entire wing, or

(2) the restriction to the black markings of the chromogen alone.

That it is the chromogen which is restricted to the black areas and the tyrosinase which is present over the whole surface is clear, for when the wing is placed in a tyrosine solution it becomes black all over, but when it is placed in a tyrosinase solution the darkening is restricted to the markings. This is similar to the case of the markings on the elytron of the Periodical *Cicada* described by Gortner [1911, 2]. Owing to the fact that the haemolymph contains a quantity of chromogen the contrast between the markings and the rest of the wing is somewhat obscured by the general darkening. On microscopical examination however the pigment of the markings can be seen within the chitin of the scales, whereas the pigment deposited on the white portions of the wing lies in irregular masses on the surface of the scales. Fortunately however it was possible to make the reaction far more marked by precipitating the tyrosinase from the haemolymph with ammonium sulphate solution and by very thoroughly washing the precipitate with the same fluid so as to remove the last traces of chromogen. If the precipitate is now dried between filter papers and re-dissolved in an equivalent amount of 0·05 % sodium carbonate solution, a fluid containing tyrosinase is obtained free from chromogen.

An attempt was made to substitute the tyrosinase prepared from the skins of young black rabbits [Onslow, 1915] but the result was not successful, owing no doubt to the difference in the tyrosinase of warm-blooded animals.

Summary.

The black markings on the wings of *Pieris brassicae* are caused by the oxidation of a colourless chromogen by a tyrosinase. This ferment is supplied from the body-lymph of the pupa, possibly by means of the wing-nervures, to the chromogen which has previously been deposited in the areas destined to become black. The form of the markings is determined by the localisation of the chromogen to these areas. The oxidation takes place just before the emergence of the fully-developed insect and as soon as the atmospheric oxygen has access to the surface of the wing.

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III. THE DISTRIBUTION OF MALTASE IN PLANTS. I. THE FUNCTION OF MALTASE IN STARCH DEGRADATION AND ITS INFLUENCE ON THE AMYLOCLASTIC ACTIVITY OF PLANT MATERIALS.

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(*Received December 17th, 1915.*)

In an investigation of the sugars present in the leaves and stems of various plants [Davis, Daish and Sawyer, 1916; Davis and Sawyer, 1916] it was found that maltose was invariably absent. Many different types of plants were examined, some of which, for example the potato, turnip, *Tropæolum* and sunflower, elaborate large quantities of starch in the leaf during the day-time to be used as a reserve during the night. If the degradation of the starch were effected merely by the ordinary diastatic enzymes, which convert starch into maltose and dextrin, maltose should be found in the leaves or circulating system of these plants, especially at night. In no single case, however, throughout the night or day could even traces of maltose be detected in starch-forming plants. Negative results were also obtained with other plants such as the mangold, dahlia and grape vine which store reserve carbohydrates other than starch (saccharose, inulin and glucose). For the first time in work of this kind a method of analysis has been used, based on the action of selective yeasts, such as *S. marxianus* and *S. exiguus* [Davis and Daish, 1913], which enables maltose to be estimated accurately in presence of other sugars such as saccharose, glucose, laevulose and pentoses. From the results of some 500 analyses, we can affirm with certainty that maltose was entirely absent from the leaf and stems of all the plants we examined. The writer is of opinion, on grounds which will shortly be stated, that this absence of maltose will be found to be a general if not universal phenomenon in plants.

From the work referred to above the writer has been led to extend the views put forward by Brown and Morris [1893] as to the mechanism of starch degradation in plants, by assuming that the enzyme *maltase* plays a part also in the transformation of starch into soluble sugars. This enzyme, instead of having a very limited distribution as assumed hitherto, probably takes part invariably in breaking down the starch in plant tissues. This degradation undoubtedly takes place in a series of successive stages, of which at least three may be distinguished:

(1) Starch ⟶ soluble starch and dextrins (*"Liquefying" enzymes*).
(2) Dextrins ⟶ maltose (*Dextrinase*).
(3) Maltose ⟶ glucose (*Maltase*).

The Presence of Maltase in Foliage Leaves and Seeds.

The fact that maltose was invariably absent from the leaves and circulating vessels of plants suggested that experiments should be made to ascertain whether maltase existed in foliage leaves, especially during the times when starch degradation is at its maximum. Such experiments carried out in this laboratory by A. J. Daish have shown that this is invariably the case; they are described in the following communication [Daish, 1916, 1].

The part played by maltase in the germination of seeds in which large stores of starch are gradually utilised has been hitherto almost entirely ignored. Since the pioneer work of Brown and Morris [1890] on the germination of some of the Gramineæ it has been always assumed that the starch is resolved by the enzymes of the seed simply into maltose and dextrin, and all calculations as to the development and activity of the enzymes in germinating seeds have been based on the assumption that a *single* reducing sugar, maltose, is formed. In the very careful work of Brown and Escombe [1898] and more recently of Stoward [1911] on the distribution of enzymes in the barley grain during germination, the fact that two saccharifying enzymes are present, capable of forming *two* sugars, has been left out of consideration. In the writer's opinion, if this fact had been recognised, certain results obtained by Stoward which are at present difficult to understand, such as the power possessed by the non-living inner endosperm of augmenting its amylolytic activity, would have found an explanation.

The results obtained by A. J. Daish [1916, 2] clearly show that in germinating barley considerable quantities of maltase are present and that the starch of the grain is resolved by this enzyme finally into glucose. We are conscious that these experiments could have been made more complete in several

directions, but as they were carried out more than two years ago, being interrupted by the war, and there is little prospect that further work can be done in the immediate future, they are published as they stand.

Maltase was first discovered in maize by Cuisinier [1885, 1886] who termed it "glucase" and patented a process of converting starch into glucose based on its use. The later work of Geduld [1891], Beyerinck [1895], Morris [1896], Huerre [1909] and Wierzchowski [1913, 1, 2] has confirmed that of Cuisinier and shown that maize is particularly rich in maltase. Regarding the presence of maltase in other cereals, especially barley, there has been a great divergence of opinion which it is now necessary to explain.

The experiments of Butkewitsch [1908] on the enzymes present in the barks and woods of plants during the spring period of starch dissolution are interesting as showing the probable presence of maltase in these parts, although Butkewitsch does not specifically recognise this. Such bark, *or even an aqueous extract of it*, hydrolyses starch and maltose; but when the extract is precipitated by alcohol the property of acting on maltose is destroyed. It is clear that this is due to the presence of *maltase*. The influence of chloroform and toluene in promoting starch dissolution is shown; when twigs are cut and stored in water containing toluene or chloroform, the starch dissolving enzymes rapidly come into action and remove the starch stored in the rind. In water alone the starch is not affected. Toluene has a far greater effect than chloroform, no doubt owing to the fact that the latter largely destroys the maltase. Butkewitsch's experiments [1908, p. 339] showing the limitation of the starch dissolving action by the presence of glucose are also important.

Failure of earlier Workers to detect Maltase in Cereals.

The failure of earlier workers to recognise the universal distribution of maltase in cereals is largely due to the fact that it is an endo-cellular enzyme which does not readily diffuse out of the plant cells and is not easily extracted by water. Extracts of the cereals (with the exception of maize) as a rule have little action on maltose and produce only small quantities, if any, of glucose, from maltose or starch. Many workers have failed to detect maltase owing to its instability. They have worked under conditions which destroyed the enzyme. Thus they used enzyme preparations which had been precipitated by alcohol or worked in presence of chloroform as an antiseptic, both of these substances *under ordinary conditions* rapidly destroying maltase.

It seems probable that *pure* alcohol and *pure* chloroform would not destroy maltase and that their usually destructive action is due to the presence

of small quantities of acid or alkali. Ordinary chloroform, when kept in a white glass bottle, frequently contains large quantities of hydrogen chloride and carbonyl chloride; when its decomposition into these substances is prevented by adding lime, the chloroform may become distinctly alkaline. Ordinary alcohol is generally acid, but in some cases, when stored in soft glass bottles, it becomes alkaline. Differences in the nature of the chloroform would explain the fact that Tebb [1894] succeeded in extracting maltase from animal tissues with chloroform water whereas Brown and Heron [1880] failed; in the latter case it is probable that the chloroform used destroyed the enzyme. In the same way the contradiction between Fischer's [1894] positive results as to the action of yeast on maltose in presence of chloroform and his later negative results [1895] under the same conditions, which confirmed the equally negative results of Morris [1895], may be explained. Similarly Beyerinck [1895] succeeded in precipitating the maltase of maize in an active condition with alcohol and Marino and Sericano [1905] the maltase of germinated barley; whilst Huerre [1909] and Wierzchowski [1913, 1, 2] state that this method gives with maize a preparation which contains little active maltase but behaves like ordinary diastase, converting starch into maltose, a result clearly due to destruction of the maltase. Our own experience [Daish, 1916, 2, p. 65] with germinated barley has been similar to that of Wierzchowski with maize.

In other cases the action on starch has been studied at a temperature of 55–60°, at which the maltase is either destroyed or at least has its activity greatly restricted. Huerre [1909] has shown that the maltase of the "*blanc hâtif*" maize of the Landes is very rapidly destroyed at 65° and somewhat less rapidly at 50° (completely in 2 hours). In other varieties of maize the maltase was found to be more resistant, probably owing to the presence of protective substances. The writer [1914] has shown that the very active maltase of taka-diastase has its power of converting maltose enormously restricted at 55°. Beyerinck's [1895] maltase from maize resisted a temperature of 55°, whilst the maltase prepared from yeast was rapidly killed at this temperature.

As a rule, the optimum temperature of plant maltases lies in the neighbourhood of 38–40° as in the case of yeast maltase; above 50° rapid weakening occurs. In some cases, however, for reasons which have not been ascertained, certain plant maltases, for example the "*haute maltase*" of maize "*jaune hâtif*" [Huerre, 1909], show a higher temperature of optimum action.

Bearing the above facts in mind it is possible to explain all the con-

tradictory statements as to the occurrence of maltase in leaves or in germinating or resting seeds. When maltase has *not* been found it has been due to the method of working either failing to extract the enzyme or destroying it. It is interesting historically to note that Brasse [1885] prior to the work of Cuisinier and Geduld on "glucase" clearly realised that the "diastase" isolated from germinated barley or from foliage leaves is capable of digesting *raw* starch to glucose, if the digestion *takes place at a low temperature* (34–42°). He points out that, providing the *extract is made rapidly in the cold* and "*subisse le moins longtemps possible l'action de l'alcool*" used to precipitate it, a product is obtained which effects the complete degradation of starch to glucose. Brasse, however, did not realise that a special enzyme (maltase) is concerned in the change as did Cuisinier shortly after, nor did he show that it acts by breaking down the maltose formed from starch by other enzymes. He realised, however, more clearly than later workers, the relative instability of the active enzyme and, in particular, points out that no glucose is formed at 50–57°. Had Brasse's paper been properly appreciated many of the contradictory statements of later workers would have been avoided.

It is not necessary here to analyse in detail the work of all those authors who failed to find maltase in plant material. Brasse, Cuisinier and Geduld considered that *all* cereals contain a glucose-forming enzyme (*glucase*). Lintner and Eckhardt [1889] contrasted the properties of the diastase of ungerminated barley with those of the diastase of malt, and although they concluded that the former gave rise only to maltose and dextrin, it would appear from the fact that they found the optimum temperature of activity (45°) to be considerably lower than that of malt diastase and a great falling off of saccharifying power to occur above 50°, that they worked with a preparation containing maltase. Later Lintner [1893] confirmed Cuisinier's statements [1885, 1886] as to the presence of "glucase" in maize and stated that glucose may also be formed in not inconsiderable quantities by the action of dried malt on starch. Brown and Morris [1893] in the case of foliage leaves observed only a transformation of the starch into maltose, and Morris [1896] stated that he had examined a considerable number of malted and unmalted grains but could find no evidence of a maltase. Beyerinck [1895] denied the general distribution of maltase in leaves and seeds (e.g. barley, wheat and rye) but found it to be present in the grain of maize, sorghum and millet. There is no doubt that in these last seeds it is especially abundant so that it could hardly escape detection even under adverse conditions of working. Later workers like Brown and Escombe [1898], Ling and Baker [1895, 1897 and

1902], B. F. Davis and Ling [1904], Ford [1904], Ford and Guthrie [1908] have all ignored the possible presence of maltase in barley and malt. On examining the conditions under which these authors worked it is clear that their negative results are to be attributed to their not realising the instability of the enzyme and the difficulty of extracting it.

Beyerinck [1895, p. 338] seems to have understood the difficulty of extracting "glucase" with water. Thus he says that rice meal contains much glucase, but on attempting to extract it he obtained, much to his surprise, an enzyme which acted strongly on starch but converted it only into maltose, without forming glucose. A similar result was obtained with oats. He is inclined to believe "though proof of this is difficult" that in the process of purification adopted, "glucase" is converted into "*granulase*" (that is, the enzyme which produces maltose and achroodextrin from starch). Obviously the maltase had been destroyed leaving behind the less sensitive ordinary diastatic enzymes.

The recent work of Wierzchowski [1913, 1, 2], published since our own was undertaken, clearly proves that all cereals contain considerable quantities of maltase, although as a rule it cannot be extracted with water owing to its endo-cellular nature. In buckwheat, millet and maize it is particularly abundant; in rye, barley, wheat and oats less so.

Wierzchowski realising the insolubility and instability of the enzyme made his experiments in the same way as we have done: the *finely-ground meal* was allowed to act upon the starch or maltose solution for 24 hours at 45° in presence of formaldehyde as antiseptic. The glucose formed was estimated as osazone; as the total reducing power was also determined, the course of action could be followed. The polarimeter was not used.

As will be seen later, Wierzchowski incorrectly interprets his results, in so far as he considers that his "maltase" could act directly upon starch, as well as upon maltose; the whole of his results, as well as our own, are best explained by the view that the maltase simply breaks down maltose which has been formed from starch by the other diastatic enzymes (see p. 39).

Presence of Maltase in Malt and Malt Extracts.

Germinated barley as Daish's [1916, 2] results show, as well as those of Brasse [1885], Marino and Sericano [1905], Marino and Fiorentino [1906] and Wierzchowski [1913, 1, 2], undoubtedly contains maltase. Whether a malt or malt extract made from such barley contains maltase or not will depend upon circumstances. If the malt be dried at a low temperature, the maltase will largely survive, but if it be kilned at a high temperature, part, if not the

whole, of the maltase may be destroyed, so that such malt will contain principally the ordinary diastatic enzymes which break down starch only to the stage of maltose. On the other hand, kiln drying, if not too prolonged or carried out at too high a temperature may actually facilitate the extraction of maltase *by water*, by rupturing the cells or destroying the protoplasm in which the enzyme is imprisoned, just as drying facilitates the extraction of maltase from yeast, which fails to yield its enzyme when in a fresh condition. These views would explain the idea of Ling and Baker [1895] that kilning confers upon malt the power of converting maltose into glucose and that air-dried malt does not possess this property; Kröber [1895] asserted exactly the opposite. It is possible to reconcile these two statements by assuming maltase to be present which was rendered more easily soluble under Ling and Baker's conditions of malting and destroyed under those of Kröber. It is equally possible to understand Morris' statement [1896] that Ling had later been unable to confirm the observations of Ling and Baker that "the ability to convert maltose into dextrose which some malts possess is conferred by the kilning process and we may therefore take it that a normal malt contains no enzyme capable of hydrolysing maltose."

Most malt *extracts* probably contain little or no maltase, owing both to the difficulty of extracting this enzyme by water and the fact that it is readily destroyed during either kilning or extraction and concentration, especially if evaporation is carried out at too high a temperature (above 45°). Brasse [1885] stated that although germinated barley contained a glucose-forming enzyme, this was entirely absent from all the commercial malt extracts he had examined.

Whilst maltase is probably absent from most commercial malt extracts this should not be presumed in the case of any sample without proof. *Fine grinding* of malt, by rupturing the cells which contain the maltase, may increase the amount of the latter which is extracted by water. Thus Wierzchowski [1913, 2] by extracting maize which was ground to different degrees of fineness obtained the following comparative results for the glucose formed from maltose:

Coarse grinding (above 2 mm.)	6·0 %	glucose
Less coarse (above 0·5 mm. sieve) ...	28·0	,,
Fine (below 0·5 mm. sieve)	37·5	,,

It is well known that the diastatic activity of a malt differs considerably with the fineness of grinding, a fact which is probably due in part to differences in the amount of maltase passing into solution.

Formation of Glucose by the action of Malt or Barley Diastases on Starch.

Ling and Baker's [1895] statement, that the diastase of high dried malt produces glucose whereas low dried malt does not, has been explained above. As a matter of fact low dried malt contains a considerable amount of maltase but it is not readily extracted. Baker [1902] working at 50° with the precipitated diastase of ungerminated barley found glucose to be formed from soluble starch after more than 6 hours; as the precipitated diastase failed to act upon maltose but converted "α-amylodextrin" into maltose and glucose, he considered that the glucose was formed from the dextrin and not from maltose. It is probable that Baker's enzyme, although it had been precipitated by alcohol and so weakened, contained some maltase, which in the long periods of digestion in his experiments gave rise to glucose. The action would in fact be similar to that of taka-diastase, which contains maltase, at 55° [Davis, 1914]; although the activity of maltase is greatly impaired at this temperature it is still capable of forming 20 % of glucose from starch after 50 hours (1 % solution of starch, 0·1 g. taka-diastase). The fact that maltose was not attacked by Baker's weakened enzyme is not surprising and may be attributed to impurities in the maltose used[1].

B. F. Davis and Ling [1904], who found glucose to be formed from starch by the action of "restricted" malt diastase, worked with a precipitated diastase prepared from a malt dried below 33°, which therefore contained undestroyed maltase; they considered that when this diastase had its activity "restricted" by heating for a short time at 60–70°, the enzyme was so modified in its nature as to give glucose among the products of its action on starch. It is more probable that the glucose owed its origin, in the prolonged periods of digestion adopted, to traces of maltase which escaped destruction during the "restriction." The slow formation of glucose under these conditions is quite parallel to the results obtained by partly destroying the activity of taka-diastase by heating at 55°. That the heating operation, which was supposed to confer the power of forming glucose, actually destroys maltase appears from the gradual falling off of the power of forming glucose with the time of heating. "Restriction" at 70·5° for example gave an enzyme which after 67 hours' action on starch gave the following quantities of

[1] Maltose recrystallised several times from alcohol in *ordinary* glass may take up sufficient alkali to destroy the very sensitive maltase. The writer has had preparations of maltose which failed to yield glucose with maltase on this account; maltose to be used in such experiments should be recrystallised in Jena glass vessels.

glucose (measured as osazone): 5 minutes' "restriction," 10·8 %; 17 minutes', 10·0 %; 30 minutes', 7·3 %; 120 minutes', 3·9 % [B. F. Davis and Ling, 1904, Series vi, vii and viii].

Ling [1907, p. 182] considers that the action here discussed was not due to maltase, as the heated diastase solution did not convert maltose into glucose; but this was probably owing to the reason given above. In their recent work on the action of precipitated diastase from kiln-dried malt on starch granules Baker and Hulton [1914] generally observed the formation of glucose at low temperatures after digestions of 4 days or more. There is little doubt that this was due to the presence of traces of maltase which escaped destruction during the precipitation of the diastase by alcohol, although they regard the glucose as formed from dextrins of low molecular weight. They consider that "it is unlikely that maltose can undergo hydrolysis by an amylase separated from malt."

Ling and Rendle [1904] stated that commercial concentrated malt extracts invariably contain glucose. Part of this glucose is probably formed from the starch of the malt by the action of maltase, but as was pointed out by Beyerinck [1895] it is probable that part arises from the inversion of saccharose, which is invariably formed in barley during germination. After extraction, the saccharose would undergo inversion by the invertase present in the extract.

The Maltase of Plants does not act directly on Starch or Dextrin but only on Maltose.

Most of the investigators who have discussed the formation of glucose from starch have considered that this sugar may be split off from the starch or dextrin molecule by the direct action of an enzyme, without the prior formation of maltose. Thus Beyerinck [1895] held that the "glucase" of maize might act directly on starch forming dextrin and glucose; also that it is capable of resolving dextrin into maltose and glucose. Ling and Baker in their various papers expressed a similar view that glucose might be formed directly from amylodextrin [compare Baker and Hulton, 1914]. Kita [1913] held a similar view with regard to the action of taka-diastase or "koji" on starch. The writer [1914] has shown that in this case glucose is undoubtedly produced from pre-formed maltose; as the glucose appears an equivalent quantity of maltose disappears and the action is perfectly continuous.

In the case of the enzymes of germinated barley it is not so easy to give a definite proof that the glucose arises only from maltose, as the dextrin

stage persists longer than with taka-diastase; as shown by Daish [1916, 2; Table VI, p. 67], dextrin is present even in the later stages (263rd hour) of the action. The fact that the starch of the barley itself undergoes slight but progressive digestion is an additional complication, it being impossible to correct for the sugars formed from the starch in a rigidly exact way by a "control" experiment as the self-digestion of the barley is greatly modified by the presence of maltose or starch [Daish, 1916, 2, p. 70]. The following considerations founded on the data in Tables VI and IX of Daish's paper [1916, 2, pp. 67, 71] make it highly probable, however, that the glucose is derived entirely from maltose and not from starch or dextrin.

TABLE I.

Action of 0·2 g. Germinated Barley on Maltose—Increments of Glucose compared with Maltose Disappeared.

Interval	Time, hours	Glucose formed, g.	Maltose calc. as equivalent to glucose formed, g.	Decrease of maltose, g.	Remarks
1	0–119	0·4268	0·4054	0·4170	Maltose corr. with glucose nearly = maltose transformed
2	119–191	0·1497	0·1422	0·1347	Maltose corr. with glucose > maltose converted
3	191–263	0·0945	0·0898	0·0615	Maltose corr. with glucose much > maltose converted

TABLE II.

Action of 0·2 g. Germinated Barley on Starch—Increments of Glucose.

Interval	Time, hours	Glucose formed, g.	Maltose corr. with glucose, g.	Maltose apparently transformed, g.	Remarks
2	119–191	0·1019	0·0968	0·0645	Maltose corr. with glucose > maltose apparently converted
3	191–263	0·0885	0·0840	0·0273	Maltose corr. with glucose much > maltose apparently converted

The tables given above show the increases of glucose (calculated as their equivalent in maltose) compared with the maltose which has disappeared in the successive intervals during the transformation of maltose and starch by 0·2 g. of germinated barley [see Daish, 1916, 2, Sections vi and vii].

As stated on p. 32 we regard the degradation of starch as taking place in a series of successive stages, each characterised by an appropriate enzyme:

Starch ⟶ soluble starch ⟶ dextrins ⟶ maltose ⟶ glucose.

All these changes run concurrently during the whole time of action, but the relative velocities of the different changes are considerably modified by alterations in the concentration of the products of action at different periods. The fact that when germinated barley is used the starch of the latter is itself hydrolysed, especially during the later stages of action, is an additional complication. In the relatively simple case of the hydrolysis of pure maltose, this factor has no great disturbing effect in the early stages of the change, as the excess of maltose largely inhibits the self-digestion of the starch [see Daish, 1916, 2, Section vii]; consequently the glucose formed corresponds with the maltose that has disappeared (actually in the first 119 hours, see Table I, the glucose formed, calculated as maltose, 0·4054 g. is slightly less than the maltose which has disappeared, 0·4170 g.). In the later stages, however, with both maltose and starch, the glucose formed, calculated as maltose, is considerably greater than the maltose apparently transformed. This is no doubt due to the fact that in these periods, as the original maltose concentration falls, more and more of the starch of the barley undergoes transformation into maltose, so that there is a new formation of maltose to replace that transformed into glucose.

That the glucose is formed entirely from pre-formed maltose even in the case of the starch hydrolysis appears clearly on plotting the data in Tables I and II, so as to show the rate of formation of glucose from starch as compared with that obtained in the case of pure maltose (Fig. 1, p. 42). With starch this curve is practically a straight line from the 119th to the 263rd hour. With maltose, the curve can be plotted from the beginning of the change and it is seen that up to about the 180th hour the rate of formation of glucose gradually falls, but subsequently the glucose is formed along a straight line which almost coincides in position and direction with that showing the formation of glucose from starch.

In no period of the starch transformation is the rate of increase of the glucose greater than that found in the maltose transformation, so that there is no evidence pointing to a direct formation of glucose from starch. In fact during the first stage, up to the 180th hour, the rate of increase of the glucose is greater in the case of the maltose hydrolysis than in that of starch as shown by the greater steepness of the curve. This is well marked from the 119th to 180th hour when the *concentration* of the glucose present is less in the case of the maltose curve than in that of starch; it is this concentration which determines the rate of formation of fresh glucose. During the period from the 180th to 263rd hour, *when the concentration of the glucose is the same in both cases,*

the curves for starch and maltose are practically coincident and form strictly parallel straight lines.

Fig. 2 shows the rate of *apparent* disappearance of maltose in the two hydrolyses. With starch the curve is less steep than with pure maltose. This is no doubt due to the fact that, in the case of starch, maltose is being formed from dextrin at the same time as it is being transformed into glucose by the maltase of the barley. Maltose therefore *apparently* disappears less rapidly in this case than in that of the maltose alone.

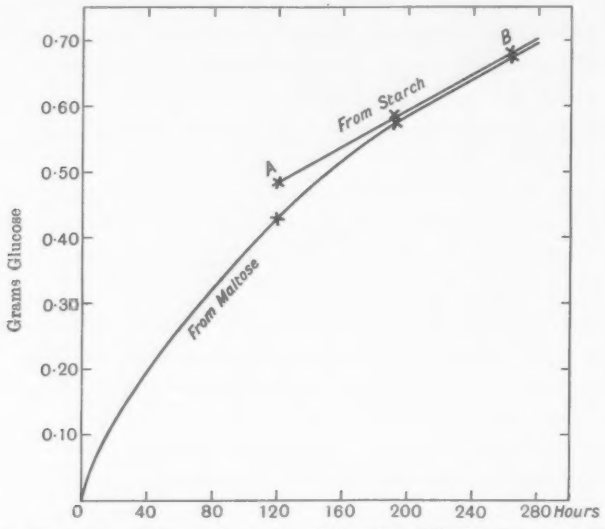

Fig. 1. Formation of Glucose by action of Germinated Barley (0·2 g.) on Maltose and Starch at 38°.

There is therefore no reason to believe that the glucose formed in the transformation of starch ever arises directly from the starch *or in any other way than by the action of maltase on pre-formed maltose.* The action of the germinated barley in this respect is exactly similar to that of taka-diastase or "koji." Beyerinck's [1895] results which were supposed to prove the contrary can be explained by the view that his precipitated "glucase," *which attacked starch and dextrin,* contained sufficient of the ordinary amyloclastic enzymes to produce the maltose on which the "glucase" could act. That this was the case appears clearly from his statement [Beyerinck, 1895, p. 335] that the formation of glucose from soluble starch or dextrins is less rapid than from maltose. In his purified "glucase" the proportion of the

ordinary amyloclastic enzymes which form maltose was relatively less than that of maltase, so that the rate of formation of glucose in the case of the starch was limited by the small rate of production of maltose. Maize indeed seems to contain the liquefying and maltose-forming enzymes in relatively small amount as compared with other cereals[1] such as barley and the maltase in relative excess, although, as the results of Wierzchowski [1913, 1] show,

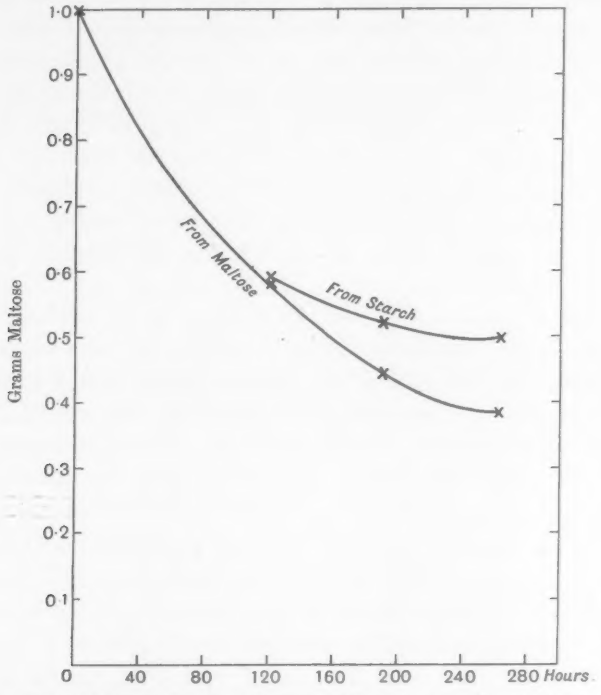

Fig. 2. Fall of Maltose by action of Germinated Barley on Starch and Maltose at 38°.

finely ground maize meal which has *not been first extracted with water* to remove these enzymes contains sufficient of the ordinary diastases to form maltose faster than it can be dealt with by the maltase.

Wierzchowski [1913, 1] commenced his work on the action of maize-maltase on starch in the hope of being able to split off glucose directly from the latter by rupturing the γ-bonds of Syniewski's formula for starch. He

[1] This appears also from Cuisinier's patent [1885]. In order to facilitate the formation of glucose from maize, the first stages of the action are hastened by the addition of *malt*, which supplies the ordinary diastatic enzymes.

found instead that action occurred, in two stages; in the first maltose was formed and in the second glucose. He correctly attributed this to the presence of the ordinary diastatic enzymes producing dextrins and maltose. In the first stage of the action, maltose is formed more rapidly by these enzymes than it can be converted by the maltase, so that it accumulates[1]. He then extracted the ordinary "diastases" from the meal, completely as he thought, by water and subjected the starch to the action of the extracted meal. He now obtained an entirely different result, glucose apparently being produced without maltose, and considered that he was effecting a pure γ-hydrolysis. The fallacy underlying this view is that in all probability he had failed *completely* to remove the ordinary maltose-forming enzymes by his preliminary extraction with water. What really happened was that these enzymes were left in relatively small proportion to the insoluble maltase present, so that when the *extracted* meal acted on starch, maltose was formed from starch far less rapidly and, *as fast as it was formed*, could be dealt with by the excess of maltase present and transformed into glucose. Consequently maltose did not accumulate. An examination of Wierzchowski's data (glucosazone yields) shows that, even in those experiments in which glucose was thought to be formed directly, some maltose was formed, so that it is clear that he had not entirely removed the ordinary amyloclastic diastases.

In the writer's opinion, there is no reason at present to assume that glucose is ever split off directly from starch or dextrins or that the degradation of starch takes place otherwise than by the series of changes:

Starch $\longrightarrow$ soluble starch $\longrightarrow$ dextrins $\longrightarrow$ maltose $\longrightarrow$ glucose,

each of which changes corresponds with the activity of a special enzyme. The localisation of the different enzymes in the seed is in accord with this view and may be briefly discussed.

Localisation of Maltase in Plant Seeds.

Brown and Morris [1890] at first regarded the scutellum of the embryo as the principal seat of diastatic activity and considered that the aleurone layer was not actively secretive; in later work Brown and Escombe [1898], however, concluded that the aleurone layer has real secretive functions, a

[1] The action of the maize meal is in these respects exactly similar to that of taka-diastase [Davis, 1914]; in both cases the breaking down of starch to maltose gives rise at first to far more maltose than can be immediately transformed. In the case of taka-diastase, dextrin disappears after the first 3 or 4 hours, and after this period the change consists solely in a transformation by maltase of maltose into glucose. The behaviour of taka-diastase towards starch under different conditions is the key to the whole question of the nature of the plant enzymes which effect starch degradation.

view supported by the recent work of Stoward [1911] which tends to show that the aleurone layer has actually a far higher amyloclastic activity than the embryo. In all this work, however, the enzymic activity has been measured on the assumption that only a single sugar—maltose—is formed during the starch transformation; the activity has been expressed in terms of reducing power only, without reference to the actual products of the action. It is unfortunate that Stoward in his recent very detailed work on the barley grain did not realise that in the depletion of the grain the enzyme maltase plays an important part, giving rise to glucose, a sugar with nearly twice the reducing power of maltose. Stoward's digestions to measure the amyloclastic power were carried out at 30°, a temperature at which the maltase could act.

Neither Stoward nor Brown and Escombe have referred to the early but very important work of Beyerinck [1895] on maize, which although giving, as we have shown above, incorrect views as to the nature of starch degradation and the general distribution of maltase, clearly indicates a different place of origin of the different enzymes in the grain. Beyerinck's work is extremely interesting as suggesting that the aleurone layer and embryo secrete in the main *different* enzymes[1]. The scutellum secretes mainly *granulase*, that is the starch-splitting enzyme, especially at the commencement of germination. The aleurone layer is the source of the "glucase," that is the maltose-splitting enzyme. Exactly the same differentiation of function is found in sorghum. According to Wierzchowski [1913, 1] also the "maltase" is mainly localised in the aleurone layer of maize, from which it is best prepared. The embryo, although active in producing the ordinary diastatic enzymes, contains little or no "glucase" (maltase); this enzyme is mainly elaborated by the aleurone cells. It is impossible in the present state of our knowledge to criticise these views, but it is highly probable that different cells secrete the ordinary diastases and the maltase of seeds during germination. It is certain that in future work on the localisation of the amyloclastic enzymes in seeds, the part played by maltase must be more fully recognised than it has been hitherto.

[1] In Beyerinck's nomenclature *granulase* is the enzyme which transforms starch into maltose and *achroodextrin*; *maltase* is the enzyme which forms maltose and *erythrodextrin*; *glucase* forms glucose (either from starch, dextrins or maltose). *Dextrinase* converts granulose into "maltodextrin" only. "Amylase" is a collective name for all the starch-splitting enzymes. Beyerinck in general names his enzymes according to the product they form: his *maltase* forms maltose, and must be distinguished from the enzyme now generally called maltase, which is capable of resolving maltose (and α-glucosides) *only* into glucose. This function is given by Beyerinck to "glucase," but as shown above this author is in error in regarding this enzyme as capable of direct action on starch and dextrin. Wierzchowski is equally in error in assuming that his "maltase" (which corresponds more closely to the modern maltase) acts directly on starch or dextrin (see p. 44).

Measurement of Amyloclastic Power (Diastatic Power) of Cereals, Malts and Diastase Preparations.

In order to obtain accurate comparative values of the diastatic activity of plant tissues or enzymic preparations it is necessary to take into account the possible presence of maltase. When maltase is present (air-dried barley, germinated or otherwise, green malts, taka-diastase, pancreas preparations) which gives rise to glucose, a sugar with nearly twice the reducing power of maltose, it is not sufficient to measure the cupric reducing power only after the transformation, but it is necessary to take into account the actual products of the change. Thus in the case of taka-diastase, which contains much active maltase, the measurement of the cupric reducing power only without reference to the specific rotatory power has led to entirely erroneous ideas as to the nature of this enzyme (e.g. by Stone and Wright [1898]). It is true that probably most of the commercial diastase preparations (malt extracts, precipitated diastases) contain very little maltase, owing to its destruction or non-extraction during the process of manufacture; but the recent work of Sherman and Schlesinger [1912] shows that pancreatic preparations, even after the processes of purification and concentration adopted, may contain a considerable amount of maltase which has escaped destruction.

With plant material such as ground barley or malt, the ordinary method of measuring diastatic activity by means of an aqueous extract probably gives a very incorrect idea of the total diastatic enzymes: this is clear from the work of Ford and Guthrie [1908] who showed that by using papain digestion in the preparation of the extracts far higher results (varying from two to sevenfold) were obtained in the case of barley. The ordinary aqueous extraction measures only the *easily soluble diastatic enzymes* which form maltose; papain extraction not only renders soluble the endo-cellular enzymes such as maltase but preserves them in solution. This preservative action is one of the principal functions of the papain in such cases; as was shown by Ford and Guthrie, high values are also obtained by using boiled papain solutions of which the digestive activity had been destroyed. How much of the increased activity in Ford and Guthrie's experiments was due to maltase is not clear: they worked at a temperature of 30°, at which maltase would be active, but they distinctly state that glucose was not formed in their experiments.

The Lintner method of determining diastatic activity at 70° F. (21° C.) would allow maltase to act, but not under optimum conditions. In Sherman, Kendall and Clarke's [1910] method on the other hand the temperature prescribed, viz. 40°, is that of the optimum activity of maltase. In the case

of diastatic agents rich in maltase, such as taka-diastase, the two methods would probably give very different results. Tests carried out at 55° at which the activity of maltase is largely destroyed would again give rise to a totally different measure of diastatic activity.

Fine grinding in the case of barley or malt, as shown on page 37, would increase the diastatic activity by liberating or rendering soluble a larger proportion of endo-cellular enzyme.

Self-digestion of Barley or Malt.

It is clear from Daish's results [1916, 2, Sections i and v] that the self-digestion of germinated barley or malt leads finally to the production of large quantities of glucose. In the early stages dextrins and maltose are also formed, but as time proceeds these give place more and more to glucose, as the action of maltase continues.

SUMMARY.

1. Maltase is probably present universally in plants in which starch degradation occurs. The failure of earlier workers to detect it is due to the fact that this enzyme is endo-cellular and therefore not easily extracted by water; and to its instability. It is easily destroyed by ordinary alcohol or chloroform. Its action, too, is greatly limited or even destroyed at temperatures above 50°.

2. Maltase occurs in considerable quantities in germinated and ungerminated cereals. In malt it may sometimes occur, if the kilning has been at a sufficiently low temperature not to destroy it. In such cases the kilning may actually render the maltase more easily extractable by water. The presence of maltase in malt or malt diastases would explain the formation of glucose from starch which has previously been attributed to other causes.

3. It is shown that the maltase of plants does not act directly on starch or dextrins but only on the maltose, which has been formed by the ordinary diastatic enzymes; no direct splitting off of glucose from starch ever occurs. When such appears to be the case, as in Beyerinck's and Wierzchowski's experiments, maltose has been formed by the previous action of the ordinary diastases and has been then acted on by maltase.

4. The action of the enzymes of germinated barley on starch is very similar to that of taka-diastase, only taka-diastase is richer than the barley in maltose-forming enzymes so that the dextrin stage is passed through more rapidly. In both cases the glucose is formed by the action of a maltase on maltose.

5. It is probable, from Beyerinck's results with maize, that the maltase is localised mainly in the aleurone layer of the endosperm.

6. In determining the diastatic activity of plant material and preparations such as taka-diastase and pancreatins, the presence of maltase should be taken into account.

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IV. THE DISTRIBUTION OF MALTASE IN PLANTS. II. THE PRESENCE OF MALTASE IN FOLIAGE LEAVES.

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In Brown and Morris' [1893] classical paper on the "Chemistry and Physiology of Foliage Leaves" the early history is traced of the question of the starch resolving enzymes present in plants. Brown and Morris proved in a conclusive manner that the starch in the leaf is dissolved by the action of a diastatic enzyme; they regarded the formation of this enzyme as a "starvation phenomenon." They showed that diastase was present in a large number of plants and concluded from a study of the sugars of the leaf, amongst which maltose was always found, that the degradation of starch is effected in the same way as by malt diastase, the end product being maltose.

As explained by Davis [1916, p. 31] the fact that maltose was never found among the sugars present in a large number of leaves examined at Rothamsted [Davis and Sawyer, 1916] led to a re-examination of the enzymes of the leaf. This has shown that in addition to the ordinary diastatic enzymes, which convert starch into dextrin and maltose, the enzyme *maltase* is also invariably present. The absence of maltose from plant leaves at all periods of the night and day is therefore easily explained. It is due to the fact that sufficient maltase is always present to convert the maltose, as fast as it is formed from starch, into the final product of hydrolysis, glucose.

It is interesting historically that Brasse [1884] in a paper cited by Brown and Morris [1893] showed that the diastatic action of leaves on starch paste at 63° gives rise to maltose[1] and dextrin just as does that of malt diastase.

[1] At 63°, the maltase was destroyed, leaving the ordinary diastatic enzymes: hence the production of maltose and dextrin [see Davis, 1916, p. 34]. At 34–42° the maltase remained and converted the maltose into glucose.

In a subsequent paper [Brasse, 1885] which Brown and Morris apparently overlooked, it is, however, shown that a "diastase" can be extracted from leaves and germinated barley *which at* 34° *to* 42° *converts starch into glucose*. As pointed out elsewhere [Davis, 1916, p. 35], Brasse realised very clearly the instability of the glucose-forming enzyme in presence of alcohol and at temperatures above 40°. He did not, however, identify it as a maltase (*glucase*), which enzyme was discovered by Cuisinier [1885] in the same year.

That Brown and Morris did not recognise the presence of maltase in leaves may be explained by the fact that, although they carried out their transformations at 30°, they used leaves which had previously been dried at 40–50° and chloroform as an antiseptic; the drying of the leaves under the conditions named would probably destroy much of the maltase whilst *ordinary* impure chloroform also renders it inactive.

The results obtained by Vines [1891] call for a word of comment. Vines showed that his leaf extracts acted on starch paste giving a cupric-reducing and fermentable sugar which he concluded was not maltose because it had no optical activity. The explanation is that Vines worked with a very large amount of leaf material so that the substance he finally obtained was a mixture of the leaf-sugars (largely consisting of invert sugar) with the products of starch transformation; by chance the positive and negative rotations of the sugars present balanced so as to give an apparently inactive product.

Robertson, Irvine and Dobson [1909] have recently stated that they found maltase as well as ordinary diastase in the leaf and root of the sugar beet. From the results given later it is clear that maltase is also present abundantly in the leaf of the closely allied mangold. This fact is very striking when taken in conjunction with the complete absence of starch from the mangold leaf during all its later stages of growth [Davis, Daish and Sawyer, 1916].

The experiments which are given below show that all the plants we have examined, which include the *Tropæolum majus* investigated by Brown and Morris, contain maltase in considerable quantities; this is true whether the leaves be plucked in the daytime or at night.

Experimental.

To avoid destroying the maltase the freshly picked leaf material was always used without drying; for the same reason toluene was used as antiseptic, not chloroform, and the starch conversions were carried out

at 38°, which is the optimum temperature for the maltase. The leaves were ground up in a mortar with a little water and the pulp was allowed to act on the starch paste or soluble starch during 24 hours or longer.

ACTION OF LEAF PULP ON SOLUBLE STARCH AT 38°.

Series I.

To 100 cc. of a soluble starch solution (= 2·2481 g. of anhydrous soluble starch dried *in vacuo* at 100° and having $[\alpha]_D^{20°} = 191·1°$) 3 g. of wet leaf previously pulped in water were added; the volume was made up to 200 cc. and after adding a little toluene was digested at 38° for 24 hours. The mixture was then boiled to destroy the enzymes, 10 cc. of alumina cream were added and the volume diluted to 500 cc. at 15°. A control experiment was made with 3 g. of leaf under the same conditions, so as to allow for the sugars in the leaf. The results are given in Table I.

TABLE I.

Leaves plucked at 11 *a.m., September* 10, 1913.

Expt.	Leaf	CuO from 50 cc. of the 500 cc. (corr.)	$a_D^{20°}$ in 200 mm. tube (corr.)	Total rotation of solution, reducing sugars calculated as	
				Maltose	Glucose
1	*Tropæolum majus*	0·1264	1·459°	1·559°	1·474°
2	Potato	0·0389	1·599°	1·668°	1·643°
3	Dahlia	0·1450	1·424°	1·536°	1·450°

The following example shows the method of calculation; the methods of analysis are those adopted by Davis and Daish [1913], the reducing power being determined under Brown, Morris and Millar's conditions.

(*a*) *Reducing sugars assumed to be entirely maltose:*

Maltose in 50 cc. $= \frac{0·1264}{1·374} = 0·0920$ g.

Therefore total maltose in 500 cc. = 0·920 g.

Starch corresponding with this $= \frac{0·920}{1·055} = 0·8717$ g.

Soluble starch taken = 2·2481 g.

Therefore starch remaining = 2·2481 − 0·8717 = 1·3764 g.

Rotation in 200 mm. tube, $a_D^{20°}$ due to maltose = 0·507°

Rotation in 200 mm. tube due to soluble starch ($[\alpha]_D^{20°} = 191·1°$) = 1·052°

Total rotation calculated = 1·559°

(*b*) *Reducing sugars assumed to be glucose:*

Glucose in 50 cc. $= \frac{0{\cdot}1264}{2{\cdot}578} = 0{\cdot}04903$.

Therefore total glucose in 500 cc. $= 0{\cdot}4903$ g. $\times\ 0{\cdot}9 = 0{\cdot}4413$ g. starch.
Therefore soluble starch remaining $= 2{\cdot}2481 - 0{\cdot}4413 = 1{\cdot}8068$ g.
Rotation due to glucose in 200 mm. tube ... $= 0{\cdot}093°$
Rotation due to soluble starch ... ... $= 1{\cdot}381°$
Total rotation $= 1{\cdot}474°$

In the case of *Tropæolum* and dahlia, which gave the highest proportion of reducing sugars and of which the diastatic activity was greatest, the rotation calculated on the assumption that the whole of the reducing sugar is glucose, is much closer to that actually observed than the value obtained assuming the sugar to be maltose. In these two cases the whole of the sugar appears to be glucose. In the case of the potato the diastatic action is far less and the calculation points to a mixture of maltose and glucose being present.

Analysis by means of Maltase-free yeasts.

Series II.

In order to make sure that glucose is formed from the soluble starch a series of experiments was carried out in which the product of transformation was analysed by means of the special yeasts (*S. marxianus* and *S. exiguus*) so as to obtain values showing the proportions of maltose and glucose actually formed [Davis and Daish, 1913].

50 cc. of a solution of soluble starch ($[\alpha]_D^{20°} = 192{\cdot}1°$), and corresponding with 1·0152 g. of soluble starch, were digested with 3 g. of wet leaf, after making the volume up to 200 cc., for 48 hours at 38° in the presence of toluene; at the end of this time 10 cc. of alumina cream were added and the mixture boiled and diluted to 500 cc. (solution *A*). For the fermentations, 150 cc. portions after adding 5 cc. of yeast-water were sterilised and inoculated with pure cultures of the yeasts. After 3 weeks, 5 cc. of alumina cream were added, the mixture was boiled and diluted to 200 cc. (solution *B*). 50 cc. portions were used for the cupric reductions (= 37·5 cc. of *A*) and the results obtained calculated back to 50 cc. of *A*.

In each experiment a duplicate control was made with 3 g. of the wet leaf so as to allow for the original sugars present in the leaf. The following table gives the corrected results.

Method of calculation: CuO from 50 cc. of *A* (corr. for leaf sugars) = 0·0888 g.
CuO due to maltose in 50 cc. of *A* = 0·0388 g.

CuO due to glucose in 50 cc. *A* = 0·0500 g., or $\frac{0{\cdot}0500}{2{\cdot}578} = 0{\cdot}0194$ g. glucose.

CuO due to maltose in 50 cc. *A* corresponds with $\frac{0{\cdot}0388}{1{\cdot}38} = 0{\cdot}0281$ g. maltose.

Total glucose in 500 cc. of *A* = 0·1940 g. = 0·1746 g. soluble starch
„ maltose „ = 0·2810 „ = 0·2664 „ „
Therefore total starch converted to sugars = 0·4410 g.
Total soluble starch taken = 1·0152 g.
Starch remaining in *A* = 1·0152 − 0·4410 = 0·5742 g.

Rotation of glucose in *A*, in 400 mm. tube ... ... = 0·082°
„ maltose „ „ ... ... = 0·310°
„ soluble starch in *A* in 400 mm. tube, taking $[\alpha]_D^{20^\circ} = 192{\cdot}1^\circ$ ... ... = 0·872°
Total = 1·264°
Actually observed in *A* = 1·222°

TABLE II.

Leaves picked at midnight, September 29, 1913.

Expt.	Leaf	CuO from 50 cc. of *A* (corr.)	CuO corr. with 50 cc. *A* after fermentation			$\alpha_D^{20^\circ}$ of *A* in 400 mm. tube	$\alpha_D^{20^\circ}$ calc. for glucose, maltose and unchanged starch
			S. exiguus	*S. marxianus*	Mean		
4	*Tropæolum*	0·0382	—	—	—	1·385°	—
5	Dahlia	0·0888	0·0364	0·0412	0·0388	1·222°	1·264°
6	Mangold	0·0873	0·0462	0·0412	0·0437	1·218°	1·280°

In the case of the dahlia and mangold, these results clearly show that more than half of the sugar produced from the starch is glucose. In the case of *Tropæolum* the reducing power was so small that fermentations were not carried out. The results given in Table III however show that with this leaf the greater proportion of the sugar formed is glucose.

It is interesting to note that the diastatic power of the *Tropæolum* and dahlia leaves picked at midnight appears to be smaller than in the case of the same leaves picked at 11 a.m. (Table I). Using the same weight of leaf in the digestion, less reducing sugars have been formed and a smaller proportion of glucose in 48 hours than in the earlier results in 24 hours. As, however, no special precautions to ensure neutrality of the soluble starch were taken and as it is well known, from the work of Ford and Guthrie and

of Sherman, that very small differences of reaction greatly influence the results obtained, no special significance can at present be attached to these data.

Action of leaf material on Gelatinised Starch.

Series III.

In this series a known weight of starch dried *in vacuo* at 130° was gelatinised by boiling for 15 minutes with 200 cc. of water; the paste was digested with 5 g. of the wet leaf during 32 hours at 38°. The solution was then boiled and made up to 500 cc. at 15° (solution *C*). In these experiments, although nearly all of the starch was liquefied, a small quantity was left undissolved in every case; on filtering the solution it was found on the filter-paper. On this account it was not possible to compare the rotation of the solution obtained with that calculated for the sugars present. Fermentations were carried out as in Series II and controls made with 5 g. of the leaf material.

TABLE III.

Action of leaf enzymes on Gelatinised Starch. Leaves picked 12 midnight, October 3, 1913.

Expt.	Leaf	Vacuum dried starch taken	CuO from 50 cc. of *C* (corr.)	CuO from 50 cc. of *C* after fermentation	
				S. exiguus	*S. marxianus*
7	*Tropæolum*	2·2340	0·0493	0·0061	0·0100
8	Dahlia	2·3250	0·2170	0·1595	0·1655
9	Turnip	2·1418	0·2110	0·1308	0·1376

In these experiments the *Tropæolum* leaf again shows a relatively low diastatic activity as compared with that of the dahlia and turnip; the greater part of the sugar, however, is seen to be glucose. The turnip leaf is particularly active, which is interesting in view of the high proportion of starch which is characteristic of this leaf [Davis and Sawyer, 1916]. In all cases the leaf enzymes have produced a mixture of maltose and glucose, the maltose generally predominating.

Action of leaves on Soluble Starch.

Series IV.

100 cc. of a soluble starch solution (= 1·6855 g. vacuum dried soluble starch, having $[\alpha]_D^{20°} = 193·7°$) were digested with 5 g. of wet leaf for 36 hours

at 38°. The solution was then boiled, 5 cc. of alumina cream were added and the volume made up to 500 cc. at 15° (solution *D*). Controls were made as usual. The calculations are similar to those of Series II.

TABLE IV.

Expt.	Leaf	CuO from 50 cc. solution *D*	CuO from 50 cc. *D* after fermentation		$a_D^{20°}$ of solution *D*	
			S. exiguus	*S. marxianus*	Measured	Calc. for sugars and unchanged starch
10	Sunflower	0·0377	0·0123	—	0·612° (100 mm.)	0·612°
11	Dahlia	0·1449	0·0460	0·0532	1·065° (200 mm.)	1·060°

In both cases, especially in that of the dahlia which has the far higher diastatic activity, the larger part of the sugar consists of glucose. The agreement between the values observed and calculated for the mixture of sugars and unchanged soluble starch is very close.

Summary.

1. It is shown that the crushed pulp of all the leaves examined (*Tropæolum*, potato, dahlia, turnip, sunflower and mangold) acts upon soluble starch or gelatinised starch, forming reducing sugars; of these the greater part consists of glucose, the rest being maltose. There is therefore no doubt as to the presence of maltase in these leaves, whether plucked at night or in the daytime.

2. When a relatively large excess of starch is used the conversion is generally incomplete. The action of the endo-cellular enzyme maltase being limited under these conditions of working, by its low solubility and low power of diffusion, maltose is nearly always found among the products.

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V. THE DISTRIBUTION OF MALTASE IN PLANTS. III. THE PRESENCE OF MALTASE IN GERMINATED BARLEY.

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It is still uncertain whether maltase, the enzyme which hydrolyses maltose to glucose, exists in germinated barley. Until recently no really satisfactory method existed of estimating glucose in presence of maltose and other sugars. Brown and Morris [1895] in a paper on the "isomaltose" of Lintner pointed out the danger of attempting to identify sugars solely by the properties of their osazones and their conclusions were supported by the results of Ling and Baker [1895, 1] with the same substance. The use of maltase-free yeasts, however, as suggested by Davis and Daish [1913] makes it possible accurately to estimate maltose when occurring with other sugars, and on this method the present work is based.

O'Sullivan [1872, 1876] showed that the sugar produced by the action of ordinary diastase on starch was not, as had generally been supposed, glucose, but maltose. Considerable confusion had in the past existed because the reducing powers of maltose and glucose were assumed to be identical; O'Sullivan showed that the reducing power of maltose is only 62 % that of glucose.

Brown and Heron [1879] also concluded that glucose is not formed by the prolonged action of malt extract on starch or maltose. It must be noted that in all their experiments the malt extract had either been heated before use to a temperature of 50° or the actual conversions were carried out at 55° or 60°. Brasse [1885], however, found that the diastase precipitated from an extract of germinated barley, providing the extraction is made

rapidly in the cold and the alcohol used in precipitating the enzyme is not left too long in contact with it, digests raw starch grains, causing corrosion similar to that found during germination and producing at 32–42° not maltose, but glucose. At 50–57°, no glucose but only maltose was formed. It is clear from these facts that Brasse was working with a preparation containing maltase, but this enzyme was not then known; no experiments were made by Brasse to show whether his enzyme acted on maltose. To Cuisinier [1885, 1886] is due the credit of realising that a special enzyme, to which he gave the name *glucase*, is abundantly present in maize and that this leads to the formation of glucose; but he did not show that the glucase converts maltose into glucose nor that the glucose formed from starch is not split off directly from the latter. It was left to Geduld [1891], working in Cuisinier's distillery, to show that glucase hydrolysed maltose to glucose. Cuisinier specially states that "glucase" is present in the organs and particularly in the seeds of a great number of plants, whether they contain starch or not, a statement which has been repeatedly denied by later workers but is undoubtedly correct.

Lintner and Eckhardt [1889] concluded that extracts of raw, ungerminated barley gave with soluble starch the same products, namely maltose and dextrin, as were obtained with malt extract but the optimum temperature of action of barley diastase was considerably lower, viz. 45°, than that of malt diastase. Brown and Morris [1890] considered that maltose and dextrin only were formed by the action of the enzymes of either germinated or ungerminated barley on starch; they always used chloroform as antiseptic in their experiments. Lintner [1893] confirmed Cuisinier's statement that maize contained an enzyme capable of forming glucose from starch and stated that dried malt might sometimes give rise to not inconsiderable quantities of glucose. Ling and Baker [1895, 1, 2] also found a small amount of glucose to be formed by the action of the diastase of *kiln-dried* malt on starch at 70° and found that such diastase formed small quantities of glucose from maltose, the glucose being approximately estimated as osazone. When *low-dried* malt was used, no glucose was formed and they considered the kilning so to modify the diastase as to confer the property of converting starch into glucose. In a later paper [1897] they modify their views and state that kilning is not the origin of the glucose-forming enzyme. Their later experiments lead them to state that glucose is *never* formed by the action of normal malt on starch. Kröber [1895] on the contrary took the view that a "glucase" existed in germinated barley but was not present after kilning. Morris [1896],

like earlier workers, found maltase in maize but no evidence of its presence in malted or unmalted barley; he states that "normal malt contains no enzyme capable of hydrolysing maltose."

Beyerinck [1895] whilst confirming the presence of "glucase" in maize denied its general distribution in plants as affirmed by Cuisinier. In wheat, rye and barley prior to germination no "glucase" could be found whilst during germination only very small quantities of glucase, if any, were formed. Beyerinck, as pointed out elsewhere [Davis, 1916], takes the view that "glucase" acts directly on *starch*, as well as on maltose, splitting off glucose directly.

Baker [1902] studying the action of the *precipitated* diastase of ungerminated barley on soluble starch found that after 6 hours at 50° only maltose and dextrins were formed; after 24 hours small quantities of glucose could be recognised by the osazone test. As glucose could not be detected after allowing this diastase to act on maltose but was formed by its action on "α-amylodextrin" he concluded that the glucose was formed from the latter and *not* from maltose. The action was regarded as not being due to maltase. At the ordinary temperature after 144 hours no glucose was formed from soluble starch, but only maltose and "α-amylodextrin." Ling and Baker [1902] found only maltose to be formed by the action of a diastase prepared from low-dried malt on starch paste at 50–60°. When the diastase was previously "restricted" by heating it above 65° its action was greatly modified and glucose was among the products of its long continued action on starch. B. F. Davis and Ling [1904] considered that the amount of glucose formed increased on raising the temperature of "restriction" until a maximum was reached at 65–70°. Glucose, however, was formed even when the enzyme had been restricted at 78°; but in this case the activity was greatly impaired. The maximum amount of glucose, estimated as osazone, was 12 % of the products of hydrolysis.

Marino and Sericano [1905] described the preparation from germinated barley dried at 25–30° of a "maltase" which hydrolysed maltose to glucose very vigorously. According to Marino and Fiorentino [1906] this "maltase" differs from the maltase of yeast in readily decomposing the β-glucosides and not the α-glucosides. In this respect this "maltase" resembled emulsin, but by a number of special experiments they considered they had shown that the property of decomposing the β-glucosides was not due to the presence of emulsin but to the "maltase" which hydrolysed maltose. No later work has been published to explain these abnormal results which contradict

the generally accepted notions as to the action of maltase and the structure of maltose. Huerre [1909, 1, 2, 3, 4] studied the properties of the maltase prepared from different varieties of maize and buckwheat. From a consideration of the differences of optimum temperature and stability towards heat he considers that a whole family of "maltases" exists, the members of which have different solubilities and different resistances to heat.

Since the present work was undertaken Wierzchowski [1913, 1, 2] has published two papers in which he shows that not only is maltase present in maize but that it exists also in smaller quantities in all cereals, for example rye, barley, wheat, oats, buckwheat and millet. In his first paper he failed to find maltase in barley or barley diastase, but in his second paper succeeded in doing so. He clearly realised that the maltase is sparingly dissolved by water, and is easily destroyed by precipitation with ordinary alcohol. He therefore worked, as we have done, not with water extracts but with the ground meal from the grain, and at a low temperature (42–45°). Wierzchowski's view that the enzyme can form glucose directly from starch is discussed by Davis [1916].

The experiments described below confirm Wierzchowski's view that germinated barley contains maltase. As it was possible to raise the objection to Wierzchowski and earlier workers whose results pointed to the presence of maltase, that the latter arose from moulds or fungi present on the grain, special precautions were taken to work with seeds which had been sterilised beforehand. To Dr H. B. Hutchinson thanks are due for valuable help. The grain was sterilised by a short immersion in a solution of mercuric chloride; after washing thoroughly with sterilised water it was allowed to germinate in sterile Petri dishes in which each grain could be observed. In no case could the growth of any mould or foreign organism be detected. The action of the germinated grain after drying and grinding was tested directly on starch and maltose at 38° in presence of toluene as antiseptic.

The results which are given show that gelatinised starch is acted on by the ground meal, the cupric reducing power of the product increasing as time proceeds whilst the rotatory power falls. The fermentations with maltase-free yeasts show that maltose and glucose are both formed, the latter increasing with time, whilst the maltose correspondingly falls. Control experiments carried out by digesting the barley alone, in which case self-digestion of the starch of the grain occurred, show that after prolonged digestion practically all the starch is converted into glucose; but in the earlier stages, considerable proportions of maltose, as well as some dextrin,

are present. When an excess of starch is used, as the maltase is relatively deficient, considerable amounts of maltose are found, but the latter is gradually transformed into glucose as time proceeds.

The results obtained with maltose show clearly that the germinated grain contains a true maltase. The action of the grain on starch is strictly comparable with that of taka-diastase [Davis and Daish, 1914; Davis, 1914] and the degradation of the starch by the enzymes of the germinated barley occurs in successive stages:

Starch → soluble starch → dextrins → maltose → glucose

[see Davis, 1916].

Experimental.

Series I. *Preliminary experiments with Unsterilised Barley.*

(i) *Self-digestion of germinated barley.* A sample of good barley, free from damaged grains, was soaked for two days in several changes of water. The seeds were then allowed to germinate in a heap on a cement floor until the rootlets were two to three times as long as the grain, the temperature being kept at 15–20°. The germinated material was dried in the air at 25–30°. Portions were then ground up in a mortar and allowed to digest in presence of 200 cc. of water at 38° for 24 hours, toluene being added (1 to 2 cc.) and the mixture well stirred at intervals. After the action the solution was boiled to destroy the enzymes, 10 cc. of alumina cream were added and the volume made up to 500 cc. at 15° (solution *A*). The weights of germinated barley represent the weights of vacuum-dried material. Air-dried material was weighed out but the weights have been reduced by the amount of water found to be present when similar material was dried to constant weight *in vacuo* at 130°. The percentage of water present was 13·2 %.

The analyses were made by the methods described by Davis and Daish [1913], the cupric reducing powers being determined under Brown, Morris and Millar's standard conditions. The values given in each case are the mean of two closely concordant results. All weights are in grams. The rotations are in true angular degrees.

For comparison of the results obtained on digestion of the barley by its own enzymes with those given by taka-diastase digestion, a similar quantity of the ground barley was heated with boiling water (200 cc.) to gelatinise the starch and destroy the enzymes. After cooling to 38°, 0·1 g.

TABLE I.

Self-digestion of Germinated Barley at 38° (24 *hours*).

Expt.	Weight of vacuum-dried germinated barley	CuO from 50 cc. of *A*	$a_D^{20°}$ of *A* in 400 mm. tube	$a_D^{20°}$ calc. for reducing sugars as maltose	$a_D^{20°}$ calc. for reducing sugars as glucose
1	4·0933	0·2605	1·076°	2·108°	0·434°
2	4·5561	0·4287	2·696°	3·475°	0·756°

of taka-diastase was added and the mixture digested 24 hours at 38°. The solution was then treated as above, 10 cc. of alumina cream added and the volume made up to 500 cc. (solution *B*). The results in Table II are corrected for the 0·1 g. of taka-diastase by a control experiment under similar conditions.

TABLE II.

Digestion of Germinated Barley with 0·1 *g. Taka-diastase.*

Expt.	Weight of vacuum-dried barley	CuO from 50 cc. of *B*	$a_D^{20°}$ of solution *B* in 400 mm. tube	$a_D^{20°}$ calc. for reducing sugars as maltose	$a_D^{20°}$ calc. for reducing sugars as glucose
3	4·0937	0·4485	1·899°	3·633°	0·798°
4	4·4948	0·4842	2·093°	3·923°	0·873°

The results in Tables I and II show that the digestion of the barley by its own enzymes produces nearly the same result as that effected by taka-diastase, which contains maltase, after destroying the barley enzymes. In all cases a mixture of maltose and glucose is formed; the rotations observed are roughly the mean of those calculated for the two sugars, showing that nearly equal quantities of these were produced in 24 hours. In the case of the self-digestion of the barley it should be noted that the starch was not previously gelatinised so that in this case the action took place on the starch granules.

In the following experiments the maltose was estimated by the use of maltase-free yeasts so that the proportion of the two sugars could be calculated.

(ii) *Estimation of maltose and glucose formed by the self-digestion of germinated barley and by the action of the barley on starch.* Approximately 5 g. of the germinated barley was ground up and digested in presence of 200 cc. of water (*a*) with 2·1210 g. of vacuum-dried potato starch, after gelatinising the latter; (*b*) without adding any starch. The digestions were continued for 48 hours at 38°, sufficient toluene being added. The solution

was then boiled, 10 cc. of alumina cream being added, and the mixture diluted to 500 cc. at 15° (solution *C*). After determining the reducing and rotatory powers, 100 cc. portions of the solution were sterilised by heating in the autoclave, after adding 5 cc. of yeast water, and fermented in the usual way with pure cultures of *S. exiguus* and *S. marxianus* for 28 days. The solutions were then boiled and 5 cc. of alumina cream added. Owing to the evaporation which had taken place in the course of the month the total volume was now slightly below 100 cc. and could be adjusted exactly to 100 cc. by adding water (solution *D*). 50 cc. portions of *D* were used to measure the cupric reducing power.

TABLE III.

Sugars formed by the Self-digestion of 5 g. of Germinated Barley and by its action on Gelatinised Starch at 38° (48 hours).

Expt.	Conditions	CuO from 25 cc. of *C*	CuO after fermentation from 50 cc. *D* = 50 cc. solution *C*		Total maltose in 500 cc. *C*	Total glucose in 500 cc. *C*
			S. exiguus	*S. marxianus*		
5	5 g. ground germinated barley + 2·1210 vacuum-dried starch	0·2938	0·2590	0·2574	1·892	1·300
6	5 g. germinated barley only	0·1194	0·0508	0·0526	0·3746	0·7256

Expt.	Conditions	Glucose / Maltose	$\alpha_D^{20°}$ observed in *C* (400 mm.)	$\alpha_D^{20°}$ calc. for maltose + glucose in *C*	Total starch from sugars[1]	Percentage of starch in barley (air-dried) digested to sugars
5	5 g. ground germinated barley + 2·1210 vacuum-dried starch	0·69	2·700°	2·631°	2·964	14·9
6	5 g. germinated barley only	1·93	0·637°	0·565°	1·008	20·2

In Exp. 5, where the germinated barley has acted on gelatinised starch, far more reducing sugars (3·192 g.) have been formed than from the 5 g. of barley alone (1·100 g.). On the other hand, in the first case the greater part of the sugar is in the form of maltose (glucose/maltose = 0·69) whilst in the second it is present as glucose (glucose/maltose = 1·93). Whilst the grain contains sufficient maltase to convert the greater part (two-thirds) of

[1] In calculating these results the sugars originally present in the germinated barley have been neglected. Strictly these are by no means negligible as the results in Section v show, but for the purposes of *comparison* and the argument of the present paper they can be ignored. The results for starch given are in reality higher than the true values by the amount of pre-existent sugars.

its own starch into glucose, when an excess of starch has been added more maltose is formed than can be dealt with by the limited amount of maltase present. The liquefying and maltose-forming enzymes are in relative excess as compared with the maltase, so that far more maltose is formed from the excess of starch than can be transformed into glucose.

In Exp. 5 the sugars formed correspond with considerably more starch (2·964 g.) than that taken (2·1210 g.), owing to the fact that the starch of the grain has been partly digested. It is probable that the whole of the gelatinised starch has been converted into sugars in the 48 hours and that the starch of the grain is undergoing a slower conversion, owing to its granular nature. This would explain the fact that the excess of the rotation observed over that calculated for the sugars, which in Exp. 5 is + 0·069° and in Exp. 6 + 0·072°, is practically identical in the two cases. This excess no doubt is due to the presence of a nearly constant quantity of dextrin in both cases: the amount of this dextrin is relatively very small, as compared with the sugars, the dextrin being transformed into sugars almost as fast as it is produced from the starch. If we calculate in Exp. 5 the quantity of starch *of the grain* which has been converted into sugars by assuming that the whole of the gelatinised starch has disappeared, it amounts to 14·9 % of the weight of the barley taken. In Exp. 6, the digested starch represents 20·2 % of the barley, showing that the addition of the gelatinised starch has arrested to some extent the self-digestion of the grain itself. The barley probably contains some 50 % of starch (see Table VII), so that in 48 hours only a small proportion of the total has been digested.

(iii) *Action of small quantities of germinated barley on soluble starch.* When small quantities of germinated barley (0·1 and 0·2 g.) are added to soluble starch considerably less conversion of the starch occurs than in Exp. 5 where 5 g. were used. After 36 hours, more than half of the soluble starch is left in the form of dextrin, whilst the greater part of the sugar remains in the form of maltose, insufficient maltase being present to effect anything like complete transformation of the latter.

In these experiments 2·17 g. of vacuum-dried soluble starch were digested with 0·1 and 0·2 g. of powdered germinated barley for 36 hours at 38°; 5 cc. of alumina cream were then added, and, after boiling, the volume was made up to 500 cc. (solution *E*). Portions of 100 cc. after adding 5 cc. of yeast water were fermented for 28 days, as in the earlier experiments, and finally made up exactly to 100 cc.

The values given show that after 36 hours' digestion 44·3 % of the soluble

starch has been converted into sugars by 0·1 g. of germinated barley, and 56·3 % by 0·2 g. of the latter. In both cases, nearly the whole of the sugar is in the form of *maltose*, the ratio of glucose to maltose in the two experiments being 0·037 and 0·077 respectively. The use of the doubled quantity of

TABLE IV.

Conversion of Soluble Starch by small quantities (0·1–0·2 *g.*) *of Germinated Barley* (36 *hours at* 38°).

Expt.	Conditions	CuO from 50 cc. solution *E*	CuO from 50 cc. solution *E* after fermentation by *S. exiguus*	CuO from 50 cc. solution *E* after fermentation by *S. marxianus*	Total maltose in 500 cc. *E*	Total glucose in 500 cc. *E*	Glucose / Maltose
7	2·17 g. soluble starch + 0·1 g. germinated barley	0·1443	0·1348	0·1349	0·9785	0·0368	0·037
8	2·17 g. soluble starch + 0·2 g. barley	0·1882	—	0·1644	1·200	0·0923	0·077

Expt.	Conditions	Percentage of starch taken converted to sugars	$\alpha_D^{20°}$ of solution *E* (400 mm.)	$\alpha_D^{20°}$ calculated for sugars only	Δ	$\alpha_D^{20°}$ calc. for sugars + unaltered soluble starch[1]
7	2·17 g. soluble starch + 0·1 g. germinated barley	44·3	2·874°	1·092°	1·782°	2·967°
8	2·17 g. soluble starch + 0·2 g. barley	56·3	2·805°	1·360°	1·445°	2·831°

barley has just doubled the ratio. The large differences between the rotations actually observed and those calculated for the sugars are due to unconverted soluble starch, as shown by the values in the last column. The differences (Δ) are strictly proportional to the starch unconverted: thus

$$\frac{1·782}{1·445} = 1·233; \quad \text{and} \quad \frac{100 - 44·3}{100 - 56·3} = \frac{55·7}{43·7} = 1·274.$$

(iv) *Attempt to separate the maltase from the germinated barley by extraction and precipitation.* Two attempts were made to separate an active preparation of maltase from the germinated barley. For this purpose a method similar to that stated to be successful by Marino and Fiorentino [1906] was used.

[1] The soluble starch used had $[\alpha]_D^{20°} = 193·7°$ and this value has been used in the calculation for the total rotation of the solution due to maltose, dextrose and unchanged soluble starch. The values actually observed are very slightly lower than those calculated. In presence of the excess of soluble starch it would appear therefore that practically none of the starch of the added grain has passed into solution. No correction has been made for the small amount of soluble sugars in the added grain.

The powdered *air-dried* grain was extracted with 1·5 times its weight of water saturated with thymol, the extract filtered and the solid matter pressed to remove as much liquid as possible. The extract was slowly dropped from a dropping funnel into eight to nine times its volume of 95 % alcohol, kept rapidly stirred. A large quantity of a slightly rose-coloured precipitate was obtained; this was immediately filtered off under pressure and dried in a vacuum desiccator. It was then redissolved in the smallest quantity of water saturated with thymol and again precipitated by dropping into alcohol. After filtering rapidly the precipitate was again dried *in vacuo*. The preparation so obtained acted very rapidly on soluble starch, but gave only maltose and dextrin as products, and no glucose. A second preparation gave a similar result. It is clear that either the method of extraction failed to dissolve the maltase or the latter was destroyed in the subsequent operations. The method described differs from that of Marino and Fiorentino in that the latter concentrated the extract *in vacuo* at 35–40° before precipitating with alcohol. They also finally dialysed their product. It is probable that differences in the nature of the alcohol may explain the differences between our results and those of Marino and Fiorentino; similar differences are to be found between the results of Beyerinck and Wierzchowski as regards the maltase of maize [see Davis, 1916, p. 34]. In any case, the negative results obtained show the instability of the maltase and the difficulty of isolating it.

SERIES II. *Experiments with Sterilised Barley.*

(v) *Analysis of the sugars present in germinated barley.* Two hundred barley grains (weight = 8·4223 g.) were sterilised in mercuric chloride solution and, after washing with sterilised water, were soaked for three days and allowed to germinate separately in sterile Petri dishes for a period of seven days. They were then dried in air at 25–30° and after grinding in a mortar were extracted in a Soxhlet extractor with 80 % alcohol for 18 hours. The extract was evaporated *in vacuo* at 35° and after the addition of 2 cc. of basic lead acetate solution made up to 100 cc. (solution *F*). 20 cc. portions were used to estimate the reducing sugars and saccharose (by inversion with 10 % citric acid); two further portions were fermented with *S. marxianus* and *S. exiguus* to give the maltose.

The values given, which must be regarded as only preliminary, show that maltose and glucose are present in nearly equal proportions; a slightly smaller amount of saccharose is present. The maltose was calculated as usual from the reducing power left after fermentation; the difference of

reducing power, 0·3149–0·1127 CuO, was assumed to be due to glucose. The sum of the rotations, 1·924°, due to the three sugars, assuming the whole of the reducing sugar other than maltose to be glucose, is slightly *less* than the value actually observed. This excludes the possibility that the reducing sugars consist largely of invert sugar. If the rotation be calculated for a

TABLE V.

Sugars present in Germinated Barley.

Expt.	Weight of air-dried barley	CuO from 20 cc. of solution *F*	CuO from 20 cc. *F* after inversion	CuO from 20 cc. *F* after fermentation with		Maltose percentage on barley
				S. marxianus	*S. exiguus*	
9	8·4223	0·3149	0·4456	0·1117	0·1137	4·91

Expt.	Glucose percentage on barley	Saccharose percentage on barley	$a_D^{20°}$ of *F* in 200 mm. tube	$a_D^{20°}$ calc. for mixture of sugars in *F*	Total sugars percentage in barley
9	4·80	3·21	2·265°	1·924°	12·92

mixture of maltose, invert sugar and saccharose it becomes + 1·325° which is much further removed from the value observed (2·265°) than on the assumption that glucose is present. It is probable therefore that the sugars are maltose, glucose and saccharose, with a trace of dextrin sufficient to cause the difference between the observed and calculated values.

(vi) *Action of germinated barley on gelatinised starch.* The sterilised barley was allowed to germinate in Petri dishes until the plumules were about $\frac{1}{4}$'' long. The grains were then removed, dried *in vacuo* over sulphuric acid and ground to a fine meal. About 1 g. of potato starch (vacuum-dried at 130°) was gelatinised by heating for 15 minutes with 50 cc. of water in a 150 cc. Jena flask and, after cooling to 38°, 0·2 g. of the barley meal was added and the mixture left to digest for various lengths of time, in presence of sufficient toluene to prevent any growth of micro-organisms, the flask being closed with a plug of cotton wool. After the digestion in each case 10 cc. of alumina cream were added, the solution raised to the boil, filtered and washed to 100 cc. at 15° (solution *G*).

20 cc. portions were used to determine the reducing power. 20 cc. portions were also mixed with 5 cc. of yeast water and fermented with the special yeasts for 21 to 25 days; after the fermentation 5 cc. of alumina cream were added, the mixture being boiled and filtered. The precipitate was washed until the volume of the filtrate was 50 cc., the whole of the latter being used for the reduction.

These results clearly show that as time proceeds the maltose present decreases in amount whilst the glucose increases. The ratio of glucose to

TABLE VI.

Action of Germinated Barley (0·2 g.) on Gelatinised Starch.

Expt.	Time of digestion hours	Weight of vacuum-dried starch	CuO from 20 cc. of *G*	CuO from 20 cc. of *G* after fermentation with *S. marxianus*	CuO from 20 cc. of *G* after fermentation with *S. exiguus*	CuO from 20 cc. of *G* after fermentation with Mean	Total maltose in 100 cc. of *G*
10	119	1·0532	0·3973	0·1598	0·1608	0·1603	0·5885
11	191	1·0192	0·4250	0·1458	0·1398	0·1428	0·5240
12	263	1·0468	0·4551	0·1363	0·1342	0·1353	0·4967

Expt.	Total glucose in 100 cc. of *G*	Glucose / Maltose	Total starch from sugars	Starch found as percentage of starch taken	$\alpha_D^{20°}$ of *G* in 400 mm. tube	$\alpha_D^{20°}$ of sugars of *G* in 400 mm. tube	Δ
10	0·4866	0·827	0·9959	94·6	5·443°	4·266°	+1·177°
11	0·5885	1·125	1·0263	100·7	4·838°	4·125°	+0·713°
12	0·6770	1·363	1·0802	103·1	4·691°	4·162°	+0·529°

maltose increases from 0·827 after 119 hours to 1·125 after 191 hours and to 1·363 after 263 hours. During the whole period part of the starch is present in solution in the form of dextrin as is shown by the value of $\alpha_D^{20°}$ observed being greater than that calculated from the glucose and maltose present. As time goes on, this difference, Δ, diminishes, being 1·177°, 0·713° and 0·529° at the different times of digestion, but even after 263 hours some dextrin is still present. The amount of enzyme taken in the 0·2 g. of barley is so small that conversion of the total starch into sugars has not been complete even after 263 hours. The action is not, however, at a standstill even after this prolonged period, as shown by the steadily increasing values of the glucose and total starch and the falling values of the maltose. The fact that the ungelatinised starch of the barley is being slowly converted into sugars accounts for the values found at the 191st and 263rd hours slightly exceeding 100 % of the gelatinised starch taken. It is noteworthy that during the last stages the increase of the glucose is greater than the corresponding fall of maltose in the same period, probably owing [see Davis, 1916, p. 42] to the rate of formation of maltose from the dextrin in solution being greater than that of the formation of glucose from maltose.

It is possible to calculate approximately at each interval the amount of the starch of the grain which has been converted into a soluble form. If this be done it is found that after the 119th hour, 33·5 % of the total weight of the added barley has been digested, after the 191st hour 37·2 %, and after the 263rd hour 38·9 %. As the actual starch present in the grain is probably about 50 %, some starch still remains undigested even after so long a period of action. For the purpose of control, experiments were made to ascertain the degree of self-digestion of 0·2 g. of the germinated barley

under exactly the same conditions as those of the experiments given in Table VI. The results are given in Table VII (solutions obtained in these controls are designated G').

It is necessary to make allowance for the pre-existent sugars in the germinated barley. The method of calculation is as follows: the values for the original sugars given in Table V are used.

		In 400 mm. tube
Maltose originally present in 0·2 g. barley	= 0·0098 producing	$\alpha_D^{20'} = +0·054°$
Glucose " " " "	= 0·0096 "	= +0·020°
Saccharose converted into invert sugar in 0·2 g. barley	= 0·0061 "	= −0·005°

Therefore maltose actually produced from starch
= 0·5885 − 0·0098 = 0·5787 g. with rotation = +3·185°

Therefore glucose actually produced from starch
= 0·4866 − 0·0096 − 0·0061 = 0·4709 g. with rotation = +0·993°

Starch corresponding to maltose = 0·5787 ÷ 1·055 = 0·5485 g.
" " glucose = 0·4709 × 0·9 = 0·4238 g.
Total starch = 0·9723 g.

Gelatinised starch not converted to sugars = 1·0532 − 0·9723 = 0·0809 g.
If this is in solution as dextrin with $[\alpha]_D^{20'} = +202°$, it produces rotation = +0·654°

Total rotation from above constituents ... = +4·901°
Actually observed rotation ... ... = +5·443°
$\Delta = +0·542°$

If Δ is due to the starch of the grain, transformed to dextrin (or soluble starch), with $[\alpha]_D^{20'} = 202°$, the concentration of the latter in 100 cc. of solution = 0·0671 g., which on 0·2 g. of germinated barley = 33·5 %.

The following table gives a summary of the results calculated in this way for the three periods of digestion.

Time in hours	Δ° due to dextrin in solution G'	Dextrin arising from grain in g.	Percentage of barley transformed
119	+0·542	0·0671	33·5
191	+0·601	0·0744	37·2
263	+0·548	0·0678	38·9

At the 263rd hour more than the whole of the gelatinised starch taken has been converted into sugars and 0·0099 g. of the starch of the barley has passed into the form of sugars. Hence the falling off in the value of Δ from the 191st to 263rd hour.

TABLE VII.

Self-digestion of 0·2 g. of Germinated Barley.

Expt.	Time in hours	CuO from 20 cc. of G'	CuO from 20 cc. G' after fermentation with *S. marxianus*	*S. eriguus*	Mean	Total maltose in 100 cc. G'	Total glucose in 100 cc. G'
10′	119	0·0506	0·0046	0·0034	0·0040	0·0145	0·0903
11′	191	0·0570	0·0034	0·0010	0·0022	0·00795	0·1062
12′	263	0·0607	—	0·0032	0·0032	0·0116	0·1114

Expt.	Glucose / Maltose	$\alpha_D^{20'}$ observed in 400 mm. tube	$\alpha_D^{20'}$ calculated for sugars	Δ	Starch corresponding with sugars	Percentage starch in barley
10′	6·2	0·345°	0·170°	+0·175°	0·0950	47·5
11′	13·4	0·241°	0·268°	−0·027°	0·1032	51·6
12′	9·6	0·255°	0·299°	−0·044°	0·1113	55·6

The above results show that the self-digestion of the barley by its own enzymes gives rise almost entirely to glucose when the digestion is allowed to proceed for a long period. On comparing these results with those of Table III and Table XII obtained with 5 g. of germinated barley it will be seen that whereas after 48 hours the ratio of glucose to maltose is 1·95, after 119 hours the maltose has practically disappeared, the ratio being 6·2; after 191 hours the ratio is 13·4 and after 263 hours 9·6. In Exp. 10′ some of the starch is still present as dextrin, $\alpha_D^{20°}$ observed being greater than $\alpha_D^{20°}$ calculated for the sugars ($\Delta = + 0{\cdot}175°$). In Exps. 11′ and 12′ this dextrin has entirely disappeared and the values of Δ are slightly negative ($- 0{\cdot}027°$ and $- 0{\cdot}044°$). This is probably due to a small proportion of the reducing sugars consisting of laevulose, which has been formed by the inversion of the saccharose present in the original grain (see Table V).

The digestion of the grain is not complete after 119 hours and is still progressing slowly even after 263 hours. The sugars formed correspond with 47·5 % of starch after 119 hours, with 51·6 % after 191 hours and with 55·6 % after 263 hours. These values for starch are probably higher than the true starch in the grain as they include the original sugars present, which amount to 12·92 % (see Table V).

If we use the values given in Table VII as a "control" to correct the values for the digestion of gelatinised starch by 0·2 g. of germinated barley we obtain the following data.

TABLE VIII.

Action of 0·2 g. Germinated Barley on Gelatinised Starch corrected for the Barley used.

Time in hours	CuO from 20 cc.	Maltose CuO (corr.) from 20 cc.	Glucose CuO (corr.) from 20 cc.	Total maltose in 100 cc.	Total glucose in 100 cc.	Glucose / Maltose
119	0·3467	0·1563	0·1904	0·5745	0·3926	0·68
191	0·3680	0·1406	0·2274	0·5150	0·4641	0·90
263	0·3944	0·1321	0·2623	0·4849	0·5550	1·14

Time in hours	Total starch calc. from sugars	Starch as percentage of gelatinised starch taken	$\alpha_D^{20°}$ (corr.) observed in 400 mm. tube	$\alpha_D^{20°}$ calc. for sugars	Δ
119	0·8982	85·3	5·098°	3·990°	1·108°
191	0·9059	88·9	4·597°	3·814°	0·783°
263	0·9591	91·6	4·436°	3·838°	0·598°

The "corrected" values show again that as time proceeds the proportion of glucose increases whilst that of maltose falls; the ratio glucose : maltose

which after 119 hours is 0·68 rises to 0·90 after 191 hours and to 1·14 after 263 hours. The proportion of starch converted into sugars rises from 85·3 % after 119 hours to 88·9 % and 91·6 % after 191 and 263 hours respectively. The difference Δ between the values of $\alpha_D^{20^\circ}$ observed and calculated for the sugars gradually falls from 1·108° at the 119th hour to 0·783° and 0·598° at the later times, as the dextrin in solution disappears owing to its transformation into sugars.

It is probable that actually the whole or the greater part of the gelatinised starch is converted into sugars, as suggested by the data of Table VI, before the more resistant starch granules of the barley are dissolved by its own enzymes, and that the latter change only makes marked progress when the gelatinised starch has largely disappeared. The self-digestion of the barley is in fact greatly modified in the earlier stages by the presence of the excess of starch so that it is incorrect to apply as a "correction" the values given in Table VII for the self-digestions carried out with the grain alone. As the gelatinised starch is transformed into sugars, the self-digestion of the added barley probably proceeds more rapidly but there is no means of ascertaining what proportion of the two kinds of starch present should figure in the calculations at the different intervals of time. As will be seen later, the self-digestion of the "controls" is greatly modified by the presence of an excess of maltose. In the latter case the effect can be more clearly traced (see p. 72).

(vii) *Action of 0·2 g. of germinated barley on maltose at* 38°. The values given above showing the transformation of starch suggest that the glucose is produced from pre-formed maltose by the action of a maltase. To test this point the action of the germinated grain on maltose was studied.

The same barley was used as in the experiments on starch. An approximately 5 % solution of maltose (recrystallised several times from 80 % alcohol) was used. 20 cc. portions of this were digested with 30 cc. of water and 0·2 g. of the finely ground barley as in the experiments with starch in presence of sufficient toluene to prevent the growth of micro-organisms. At the end of the digestion 10 cc. of alumina cream were added to each flask and the contents were boiled to destroy the enzymes. After filtering, the precipitate was washed until the volume of the filtrate was nearly 100 cc., and the solution was finally made up exactly to 100 cc. at 15° (solution *H*). 20 cc. portions of the filtrate were used to determine the reducing power and 20 cc. portions were fermented with *S. marxianus* and *S. exiguus* as in

the starch experiments. 20 cc. of the original maltose solution were diluted to 100 cc. and the reducing power and rotatory power determined with 20 cc. portions (+ 30 cc. water) so as to obtain values comparable with those of the digestions. That prolonged digestion with water alone has no action on maltose was shown by the following experiment. 1·006 g. of the maltose was added to 50 cc. of water and after adding 5 cc. of toluene the mixture was left in a flask closed with cotton wool for 165 hours at 38°. 5 cc. of alumina cream were added, the solution was boiled and made up to 100 cc. at 15°. The rotation of the original solution was 5·290° in a 400 mm. tube at 20°; after the prolonged digestion and treatment as above, the rotation was 5·291°.

TABLE IX.

Action of Germinated Barley (0·2 *g.*) *on approximately* 1 *g. Maltose in* 50 *cc. of water at* 38°.

Expt.	Time in hours	CuO from 20 cc. of solution *H*	CuO from 20 cc. of solution *H* after fermentation with			Maltose in 100 cc. *H*	Glucose in 100 cc. *H*
			S. marxianus	*S. exiguus*	Mean		
13	Nil	0·2722	—	—	—	0·9980	—
14	119	0·3672	0·1580	0·1585	0·1583	0·5810	0·4268
15	191	0·4006	0·1184	0·1247	0·1216	0·4463	0·5765
16	263	0·4262	—	0·1048	0·1048	0·3848	0·6710

Expt.	Glucose / Maltose	Total sugars in solution calc. as maltose	Percentage of maltose left unchanged	$\alpha_D^{20°}$ of solution *H* in 400 mm. tube		
				Observed	Calc. from sugars	Δ
13	—	0·9980	100·0	5·300°	—	—
14	0·735	0·9860	58·2	4·403°	4·098°	+0·305°
15	1·292	0·9939	44·8	4·035°	3·671°	+0·364°
16	1·744	1·0223	38·6	3·763°	3·533°	+0·230°

If we "correct" these values by the control experiments carried out with 0·2 g. of germinated barley (Table VII) we obtain the following data (Table X).

The results given in Table IX are very striking. The values for maltose and glucose are calculated from the reductions before and after fermentation in the usual way. The maltose is seen to fall steadily whilst the glucose increases. The ratio glucose : maltose rises steadily from 0·735 after 119 hours to 1·744 after 263 hours. From the values given in the 10th column for the total maltose corresponding with the sugars in solution[1] it is seen that during

[1] Calculated as follows, for example for the 119th hour:
Total sugars as maltose = 0·5810 g. + (0·95 × 0·4268) = 0·9860 g.

nearly the whole of the action these sugars correspond only with the maltose taken; practically no sugar has been formed from the starch of the barley. *Thus in presence of the excess of maltose the self-digestion of the barley, which gives rise to the values in Table VII, has been almost entirely suppressed.* It is only in the last stage of the action (263rd hour) that the total sugars expressed as maltose (1·0223 g.) exceed the maltose originally taken (0·9980 g.); even here the excess is small, but there is no doubt that in these latter stages additional sugar is being formed from the barley.

TABLE X.

Hydrolysis of Maltose by 0·2 g. Germinated Barley, "corrected" for the Barley used.

Expt.	Time in hours	CuO from 20 cc. *H'* (corr.)	Glucose CuO from 20 cc. of *H'*	Maltose CuO from 20 cc. of *H'*	Glucose in 100 cc. of *H'*	Maltose in 100 cc. of *H'*
13′	Nil	0·2722	—	0·1996	—	0·9980
14′	119	0·3166	0·1623	0·1543	0·3315	0·5650
15′	191	0·3436	0·2242	0·1194	0·4630	0·4383
16′	263	0·3655	0·2639	0·1016	0·5510	0·3730

Expt.	Glucose / Maltose	Percentage of maltose left unchanged	$\alpha_D^{20°}$ (corr.) in 400 mm. tube: Observed	Calc. for sugars	Δ	Total sugars calc. as maltose
13′	—	100·0	5·300°	—	—	0·9980
14′	0·586	56·7	4·058°	3·809°	+0·249°	0·8799
15′	1·057	43·9	3·794°	3·389°	+0·405°	0·8781
16′	1·478	37·4	3·508°	3·215°	+0·293°	0·8955

A comparison of the values of $\alpha_D^{20°}$ calculated for the sugars with those actually observed shows that up to the 191st hour the observed rotation is in excess of that calculated by a *nearly constant* but slightly increasing amount (+ 0·305° to + 0·364°); this is no doubt due to the formation of dextrin from the starch of the 0·2 g. of barley, the dextrin increasing only slightly in amount and undergoing very little if any transformation into maltose in the earlier stages. During the last stage of the action (191st to 263rd hour), when the concentration of the original maltose has greatly fallen, the dextrin from the barley starch apparently begins to be transformed into maltose, as shown by the value of Δ diminishing from 0·364° to 0·230°, whilst the total sugars expressed as maltose slightly exceed the original maltose taken.

The above results show that when working with enzymic material, such as germinated barley, a control experiment carried out with this material and water alone does not always give values which can be applied as a true correction, owing to the activity of the enzyme being greatly modified by the

presence of the substrate the change of which is being studied. From Table X it is seen that whilst the maltose values are only slightly changed by introducing a "correction" for the 0·2 g. of barley, since the maltose values obtained with the latter are in themselves very small, the results for glucose are very different from those in Table IX, and far lower than they should be. The sum of the sugars calculated as maltose is always far less than the original maltose taken (viz. 0·8781 to 0·8955 instead of 0·9980), the difference being greatest in the early stages of the action when the effect of the maltose in limiting the self-digestion of the starch is most pronounced. It is clear that the "correction" introduced in this way is far greater than that corresponding with the actual self-digestion of the starch under the modified conditions of experiment.

The formation of glucose from maltose takes place even in short times of digestion. The following series of experiments was carried out to ascertain whether glucose can be detected in the earliest stages of the action. The details of the experiments were the same as in those described above but the times of digestion were shorter.

TABLE XI.

Action of 0·2 *g. Germinated Barley on Maltose Solution at* 38°.

Expt.	Time of digestion hours	Mean CuO from 20 cc. of solution	$\alpha_D^{20^\circ}$ observed in 400 mm. tube
17	Nil	0·2746	5·36°
18	24	0·2857	4·98°
19	48	0·2927	4·92°
20	145	0·3395	4·04°

In these experiments no fermentations were made to ascertain the proportion of maltose and glucose present. It is clear, however, from the increase of reducing power and the fall of rotatory power that the glucose is formed from the start. Fig. 1 shows the result obtained in plotting the values of CuO and $\alpha_D^{20^\circ}$; both the reducing power and the polarisation appear as linear functions of the time.

(viii) *Self-digestion of* 5 *g. of germinated barley.* 5 g. of the barley were ground up and digested at 38° with 100 cc. of water in presence of toluene during 48 hours. 10 cc. of alumina cream were then added, the solution was raised to the boil, filtered and made up to 500 cc. (solution *K*); 50 cc. portions were used for the direct measurements of cupric reducing power and similar portions were fermented as usual with the special yeasts.

After 48 hours' self-digestion of the barley at 38° roughly two-thirds of the sugar in solution are in the form of glucose (glucose : maltose = 1·95). This experiment has given almost identical results to those with 5 g. of the unsterilised barley in Exp. 6, Table III (glucose : maltose = 1·93). In both

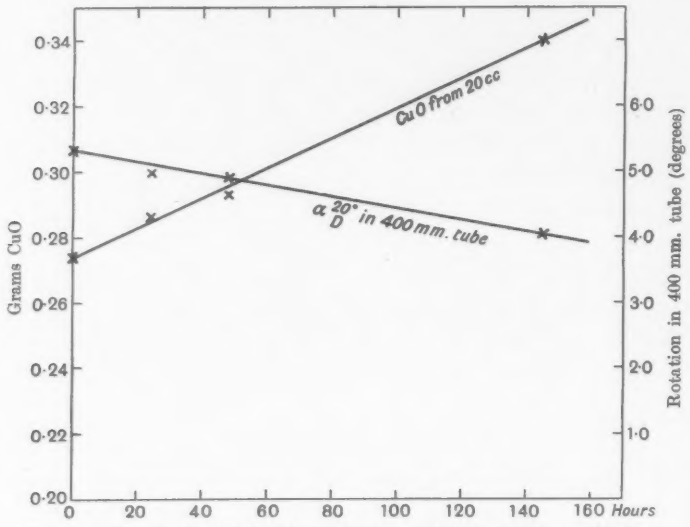

Fig. 1. Action of 0·2 g. of Germinated Barley on Maltose at 38°. (Table XI.)

TABLE XII.

Self-digestion of 5 g. of Germinated Barley.

Expt.	Mean CuO from 50 cc. of solution *K*	CuO from 50 cc. *K* after fermentation with			Glucose in 500 cc. *K*
		S. marxianus	*S. exiguus*	Mean	
21	0·2388	0·0526	0·0508	0·0517	0·7395

Expt.	Maltose in 500 cc. *K*	Glucose / Maltose	Starch in the form of sugars	Percentage of starch calc. on barley
21	0·3794	1·95	1·0252	20·5

cases practically the same proportion of the starch of the grain has been digested: in Exp. 6 the sugars calculated as starch represent 20·16 % and in Exp. 21, 20·5 % of the barley taken. This does not represent the total starch of the grain as shown by the results in Table VII, but only the amount digested in 48 hours. In the above calculation no allowance has been made for the pre-existent sugars in the germinated barley.

SUMMARY.

1. It is shown that air-dried germinated barley contains a *maltase* which hydrolyses maltose to glucose. Attempts to separate this enzyme by extraction with water followed by precipitation with alcohol caused its destruction; the preparation obtained only acted on starch in the same way as ordinary malt extract, forming maltose and dextrin.

2. The presence of maltase in germinated barley is shown, however, by allowing the finely powdered grain to act upon starch or maltose at 38°. Under these conditions a large amount of glucose is formed in both cases; as the proportion of glucose increases, that of the maltose falls. There is no doubt that in the case of the starch the glucose arises wholly from preformed maltose and not directly from starch. The action on starch is very similar to that of taka-diastase, which contains maltase in addition to the ordinary diastatic enzymes.

3. When the germinated barley is allowed to digest itself for a prolonged period, practically the whole of the starch disappears and is converted into glucose. In the earlier stages of the action dextrin and maltose are found but they gradually disappear until only glucose remains. The action probably takes place in the series of stages:

starch → soluble starch → dextrins → maltose → glucose.

4. When a small quantity of germinated barley (0·2 g.) is used to digest gelatinised starch or maltose, the self-digestion of the barley starch is largely inhibited until the greater part of the added starch or maltose has been converted into glucose. This makes it impossible to apply a "correction" for the enzymic material by carrying out a control with the latter in presence of water only.

5. When the germinated barley (0·2 g.) digests gelatinised starch, dextrin, maltose and glucose are found even after very prolonged periods. The dextrin and maltose gradually and continuously fall, but even after 263 hours both are still present; the glucose steadily increases in amount during the whole period of digestion.

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VI. OBSERVATIONS UPON THE GROWTH OF YOUNG CHICKENS UNDER LABORATORY CONDITIONS.

By JACK CECIL DRUMMOND.

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(*Received December 24th, 1915.*)

There are not many published records of the use of young chickens as the experimental animals in studies upon the factors concerned in growth and nutrition, but, from a reference to those of recent date, the present author was led to believe that one might rely to some considerable extent upon their suitability for such studies.

When the present work was begun, it was intended to make a full study of the effect of various diets upon tumour growth in the young, growing chick.

The particular tumour which was selected for use was one of the avian neoplasms described by Rous [1910]. This growth, originally found by him in a Plymouth-rock hen, simulates in many respects a spindle-celled sarcoma. It is very suitable for the study of the effect of diet upon tumour growth, on account of its high degree of virulence, its rapid growth, and its great tendency to form metastatic deposits in other parts of the body.

Peculiar interest is also attached to the growth from the fact that it may be transmitted by means of the cell free filtrate from the crushed tumour. For this reason, as well as on other grounds, many workers consider that the growth should be classified as one of the infective granulomata, rather than as one of the true sarcomata.

Whether this is so or not, is a matter which remains as yet undecided. Meanwhile it was felt that the tumour sufficiently simulated a true sarcoma to warrant an investigation along the lines previously mentioned.

At the outset of the work, Dr A. Paine, the Director of the Research Institute, brought to my notice an observation that he had made. He had

been struck by the poor standard of development and nutrition reached by large batches of young chickens reared in the Laboratory animal room from an early age, and, in view of some of the published results from this Laboratory, it was felt that the matter was of sufficient importance to justify an investigation.

It will be useful if reference is made here to some of the earlier research which has been done upon the growth of young chickens and upon the effect of various dietaries upon the growth of Rous' sarcoma grafts which they carried.

Funk [1913] utilised, for a research of this nature, young chicks of the Plymouth-rock strain, which were a fortnight old when observation was begun upon them. They were divided into different batches, which were fed upon different variations of a rice diet. After the elapse of 15 days, each bird received an injection of Rous' sarcoma, and the effect of the diet upon the subsequent development of the tumour graft was then noted.

He found that the young birds, which received as their sole food cooked polished rice, invariably died after some fourteen days without exhibiting any apparent symptoms of beri-beri.

A further series of birds were fed entirely upon cooked "red" (unpolished) rice, and results of apparent importance were obtained upon this batch.

It was found that the chicks remained alive for some four weeks or so without manifesting any sign of further growth. During this period, Funk's control birds, fed upon chicken food, doubled their initial weight.

There is no need to deal here with the results of the dieting upon the injections of tumour material. They are strictly in accordance with the well-known fact that tumour grafts will not flourish well in an ill-nourished host.

Funk apparently kept observation upon his young chicks for a period of only twenty-eight days, a period, however, that he considered sufficiently long for his purpose, in spite of the fact that he realised to some extent the difficulty of rearing the little birds upon such monotonous diets. It is noteworthy that Funk did not observe any appreciable diminution of the appetites of the experimental animals.

In a later publication Funk [1914] corroborates his previous finding, that it is possible to arrest the growth of young chickens by feeding them upon a diet of "red" rice. A photograph is contained in his paper which contrasts very plainly the great difference in size and development of two chickens, one of which had been fed entirely upon a diet of "red" rice, while the other had received normal food.

Subsequently, in collaboration with Macallum, Funk [1914] published the description of extended studies upon the arrest of growth in young chicks. By adding a small ration of cod-liver oil to the diet of "red" rice, Funk and Macallum claim to be able to arrest the growth of young chicks over a much greater length of time than had been previously done upon a diet of "red" rice alone. They give somewhat detailed particulars of an individual bird which was maintained at an almost constant body-weight for a period of no less than five months by this means.

There is also appended to their communication another apparently striking photograph of this particular chick, standing by the side of a normal bird of the same age. It is not, however, stated whether the normal bird, whose photograph is reproduced, was reared in the Laboratory, and from the same series of chicks as the stunted one. In view of the results which will be shown in the present paper this question is one of the greatest importance.

The stunted chick possessed several peculiarities which were noted by Funk and Macallum. They found that the bird failed to develop any of the secondary sexual characteristics of the fowl, its voice is described as that of a four-week-old chick, and apparently the growth and development of the beak and feathers were not retarded to such a degree as was that of the body itself.

The present writer was so much impressed with the importance of the possibility of being able to stunt at will, for comparatively long periods of time, the growth of the young chick, that the preliminary experiments were directed to corroborate this finding, if possible.

Accordingly, in the early summer months of 1915, a large batch of young Plymouth-rock chicks was obtained from a reliable breeding farm in the country. The young birds arrived at the Institute at the age of ten days, and they were at once placed in a heated foster-mother where they were reared until somewhat older and stronger.

This was done as several preliminary experiments had shown that very young chicks are too delicate to be fed with unnatural dietaries.

The birds were strong and healthy when brought in, and, during the whole of the time that they were being hardened off in the foster-mother, they ate their food well. They were weighed regularly, and observation both of these weights and of their general appearance warranted the assumption that they would develop into healthy vigorous chickens.

It might be mentioned here that the foster-mother was constructed of wood, and the heating regulated so that as time elapsed the young birds

might be gradually hardened off. The floor was covered with a layer of fine sand and grit.

The diet supplied to the birds during this period was mainly a special chick-food, chopped egg and bread crumbs, and fresh green food.

After having been in the foster-mother for a month, the birds were divided into four batches, each of which was placed in a separate pen. The pens were large and well ventilated, provided ample room for exercise, and possessed a covered-in end, fitted with a warm sleeping shelter. The floors of the pens were covered with coarse sand, shell mixture and sawdust. The animal-room, in which the pens were kept, is large and airy, and has been constructed upon scientific principles for the housing of experimental animals under as natural conditions as possible.

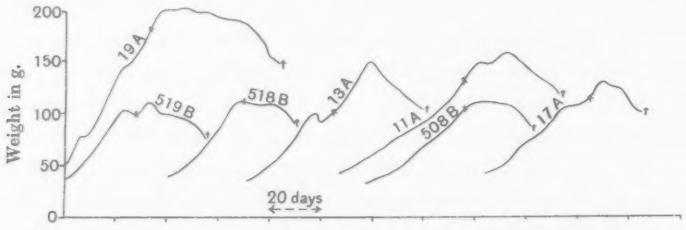

Chart I. Growth curves of chicks fed upon a diet of cooked "black" rice. × marks commencement of diet.

At this point in the investigation all the young birds were in good health as far as could be judged. They continued to eat well and the weekly increment of body-weight was satisfactory. All four batches were fed normally in the pens for a fortnight, receiving chicken-food, corn, meal and fresh green vegetables.

It was towards the end of this fortnight that the first cases of indisposition occurred. One or two of the chicks showed signs of weakness, particularly in the legs, and in these cases it was noted that the increase in body-weight was not as satisfactory as it had been.

Particular notice was not, however, paid to these birds, as it was considered that they might be exceptional cases.

They were accordingly weeded out, and the remaining chicks were placed upon various diets. One group received as sole source of nutriment cooked polished rice, a second was fed entirely upon cooked "red" (unpolished) rice, a third batch was given another variety of unpolished rice, namely, "black" rice, in the cooked state, whilst the fourth and last group was maintained on normal food. Daily observation was made on the birds,

and they were weighed at intervals of four days. The birds which were fed upon "white" polished rice behaved in the manner that was expected. They almost immediately failed to register an increase in body-weight, then followed a period of rapid decline and finally death (Chart II). Failure of appetite did not seem apparent during the decline, until in the latter stages. There is little need to comment upon the results obtained in this series of birds, for the results are but a corroboration of those which have been published by a considerable number of workers.

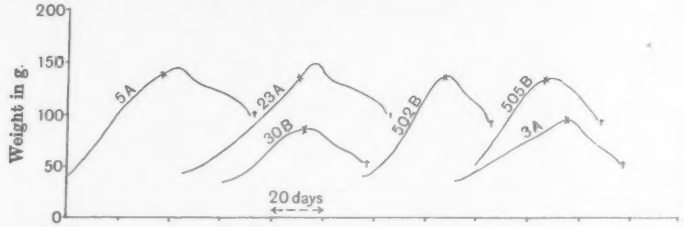

Chart II. Growth curves of chicks fed upon diet of cooked "white" rice.
× marks commencement of diet.

The second group of birds, which were given "red" rice alone, were watched with particular interest in view of the findings of Funk which have been referred to.

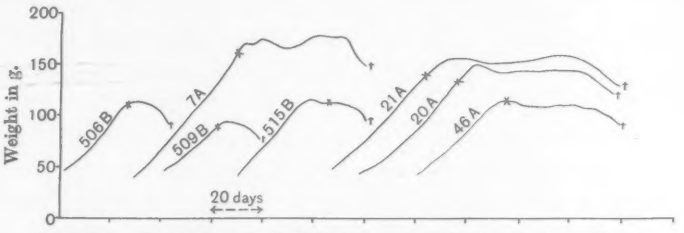

Chart III. Growth curves of chicks fed upon diet of cooked "red" rice.
× marks commencement of diet.

It was found, on an examination of the body-weights of the chicks in this batch, that growth was in many cases suspended shortly after the commencement of the feeding. Many of the birds did maintain an almost constant body-weight for periods of time varying from twenty to eighty days (Chart III). It was, however, soon obvious that the chicks were far from being in a healthy condition. The observations of Funk and Macallum with regard to the absence of any development of the secondary sexual characteristics and of the voice, and the comparative overgrowth of beak and feathers of these chicks, were confirmed. Many of them showed a

weakness in the legs and they all presented a ruffled appearance. Again, as in the case of the animals fed upon "white" rice, no apparent failure of the appetite was observed until a few days before death, which usually occurred after a rapid decline, following the period during which maintenance was marked.

Post-mortem examination of the chicks which died in this batch failed to reveal many apparent abnormalities. The birds were emaciated and there was a poverty of blood in the organs, the majority of which were atrophic; particularly was this the case with the thymus and the spleen.

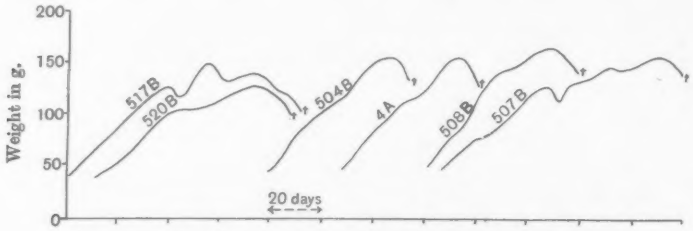

Chart IV. Growth curves of control chicks, showing early failure.

The third series of chicks, fed upon "black" rice, reacted to the diet in a very similar manner to those which had received "red" rice. This is a matter for no surprise when one considers that the two diets are very similar. If anything, the period during which growth was stunted was shorter, and the final decline more rapid than in the case of the chicks fed upon "red" rice (Chart I).

The really interesting results of the work, however, followed, strangely enough, from a study of the remaining batch of chicks which had been retained upon normal diet, to act as control animals to the other three groups (Chart IV).

Mention has already been made of the one or two cases of weakness which were observed before the chicks were placed upon special diets.

It was now found that further similar cases occurred with increasing frequency amongst the control birds. Taken collectively, the batch began to present the same ruffled appearance and weakness in the legs as those which were fed solely upon rice diets, while few of them continued to put on weight.

Only one particularly strong chick continued to grow at a rate at all approximating to normal. There were, on the other hand, several cases of arrest of growth over considerable periods of time, followed occasionally by a slow resumption of a very subnormal rate of growth, or, as was the case

with the majority, by a rapid decline and death (Pl. I, photo. 2). Few of these birds lived longer than 120 days, which is little better than those which had been fed solely upon "red" rice.

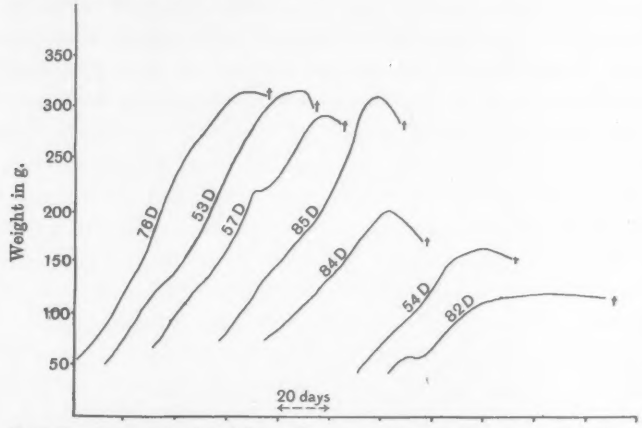

Chart V. Curves showing failure of young chicks reared upon earth surface.

Even the one bird referred to above did not maintain its superior rate of growth for long. After fourteen weeks it arrived at its maximum body-weight of 500 g. and it was then a fairly strong chick (Pl. I, photo. 1). Subsequently it maintained its body-weight for some few weeks after which it declined in the same way as had done the other birds of this group.

The results obtained in the series of experiments may be tabulated in the following form:

No. of chicks in batch	Diet	Average length of life, days	Average maximum body-weight attained, g.	Average weight at death, g.
12	normal	123	256	216
12	"white" rice	65	107	91
12	"red" rice	82	167	100
12	"black" rice	76	162	113

From these average results it will be seen that the consequences of feeding "red" rice to young chicks is not much more detrimental to the nutrition of the animal than if one retains the bird on normal food.

The failure of the normal birds to grow and develop was at first attributed to possible defects in the condition of keeping or feeding. Thus, poultry-keepers sometimes consider that weakness in the legs is due to a deficiency of fresh meat in the diet. It was therefore attempted to rear a batch of young chicks upon normal food, giving them in addition a ration of fresh

meat daily. The birds ate the meat greedily, but did not respond to the change, and the effort to raise them was no more successful than before.

It was then thought that if the young birds were reared upon a more natural surface they might thrive more satisfactorily. Accordingly, two batches were reared separately, one being brought up upon a surface of sawdust and coarse sand, scattered on the floor of the cage (Chart VI); whilst the other group was provided with a surface of fresh earth and grass, which was frequently changed (Chart V).

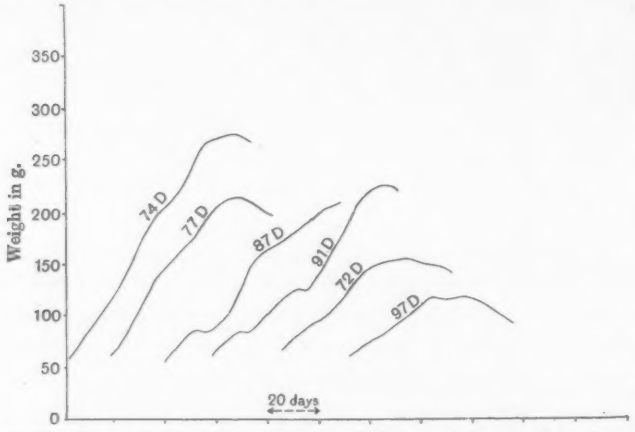

Chart VI. Curves showing failure of young chicks reared on a surface of sawdust and sand.

Observation upon both batches was made as before, but very little improvement was noted in those reared under the more natural conditions. Certainly, as is seen from the tabulated results, they lived on the average somewhat longer, and attained a slightly greater maximum body-weight, but the usual symptoms of weakness, which have been described, made themselves apparent sooner or later and the chicks declined and died.

No. of chicks in batch	Reared upon	Average length of life, days	Average maximum body-weight attained, g.	Average weight at death, g.
12	Sand and sawdust	80	173	168
12	Earth and grass	93	239	229

Post-mortem examination of a large number of these "normal" chicks did not bring to my notice any particular point of interest. The bodies were without exception greatly emaciated. The organs were usually small and anaemic. During life this apparent poverty in blood was striking. The

combs and eyelids of the chicks were peculiarly bloodless, being of a dead white colour, as contrasted with the deep red colour possessed by the combs and eyelids of normal fowls of an equal age.

The results were of so striking a nature, and of such great importance in a consideration of the value that should be placed upon results of previous investigations carried out upon young chickens, that the experiments which have been described were repeated upon a further batch of young Plymouth-rock chicks.

This series were hatched out from eggs in the Institute, and were thus under observation from their birth. They were a strong healthy brood when born, and they were carefully reared in the foster-mother until five weeks old. They were then divided into similar batches to the other series, placed out in pens, and fed upon similar dietaries.

The results obtained were identical in every way with those which have already been described. The onset of the symptoms of weakness was, however, earlier in every case, and consequently the birds did not live so long or attain so great a weight.

No. of chicks in batch	Diet	Reared upon	Average length of life, days	Average maximum weight attained, g.	Average weight at death, g.
10	normal	earth	81	202	171
10	normal	sand	74	187	165
10	"red" rice	,,	45	103	87
10	"white" rice	,,	41	108	80
10	"black" rice	,,	47	112	80

There now seemed every reason to believe that the observation communicated to me by Dr Paine was substantiated by facts, and that it was indeed a matter of difficulty to rear young chickens at all under the artificial conditions of an indoor animal-room.

While this work had been in progress, it was observed that a batch of Plymouth-rock hens which had been brought into the Institute at the age of six months were continuing to grow at a normal rate and appeared in every way healthy and strong. This observation suggested that an investigation into the effect of the age at which the young chickens were brought into the unnatural surroundings upon their subsequent development might yield useful data, particularly as large numbers of full-grown birds have been kept for long periods of time, under the same unnatural surroundings, without in any way showing any tendency to decline. Even egg production occurs in these full-grown fowls at a normal rate.

For the purpose of this research a large number of young Plymouth-rock

chicks were hatched out in the country and were then brought into the Institute after various periods of time. Observation was kept on them closely after they had been brought into the artificial conditions and the results have been tabulated as follows (Chart VII):

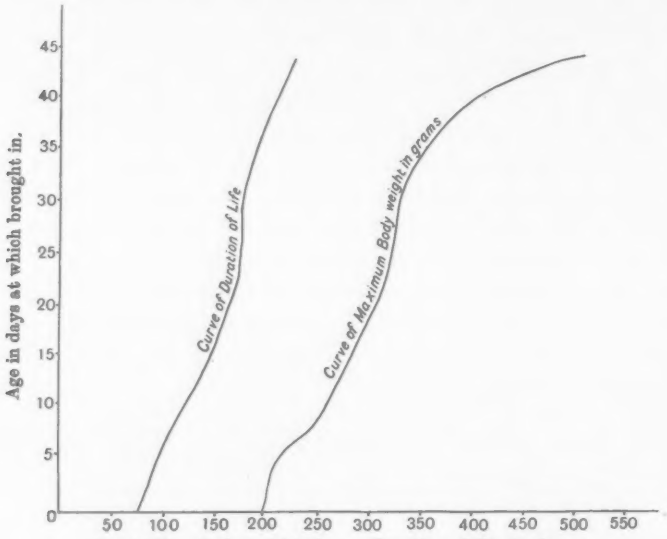

Chart VII. Body-weights in g. Duration of life in days.

No. of chicks in batch	Age brought in, days	Average length of life, days	Average maximum body-weight, g.	Average weight at death, g.	Remarks
20	0	77	197	168	All died exhibiting signs of extreme malnutrition
16	4	86	206	198	ditto
25	7	84	233	184	ditto
12	10	123	256	216	ditto
12	21	152	307	290	Symptoms not so severe, yet all died from malnutrition
8	28	160	320	300	ditto
10	42	6 yet alive after 205 days	497	—	Poorly nourished and ill developed. Six yet alive, but all declining rapidly

The ten birds in the last group have resisted the unfavourable influences of the unnatural surroundings longer than any other batch have done. They have never presented, even at the time when they registered a maximum body-weight, a strong appearance.

During the period of decline they have not shown the symptoms of

weakness to anything like the same extent as did the first four batches, nor did they possess the pallor of the comb and eyelids which was so characteristic of the earlier groups of birds.

We now come to a consideration of the results which have been obtained. Firstly, it is felt that the young chick is not a suitable subject upon which to conduct experiments in the study of growth, at any rate, when it is kept under artificial conditions. The difficulty of rearing young fowls indoors is, I believe, realised by poultry-farmers generally, so much so in fact, that it is seldom attempted by them.

An explanation of the cause of the failure has not yet suggested itself. No dietary deficiency can be held as responsible, for the diet supplied to the experimental chicks in the normal batches was in every way similar to that successfully employed to rear chicks in the open air. An attempt to supply one of the conditions, under which fowls are normally reared, was made when fresh earth and grass were given to provide a surface for the young birds to run upon.

They certainly made use of the soil, scratching it over and picking out small insects and other tasty morsels all day long. And yet, despite the fact that the earth was changed frequently, this group of birds reached but a slightly better standard of development than did a similar batch, which had a surface of sawdust and grit to run upon.

In the light of these facts we must admit that there are other factors than an adequate diet, which must be supplied to the growing chick, in order that it may complete its normal growth when under conditions such as are described.

If we submit the results which have been published by Funk, and by Funk and Macallum, upon growth in young chicks, to a criticism directed along these lines, we are forced to conclude that they now have a much smaller value.

It is unjustifiable to deduce results from the feeding of an experimental animal upon a certain diet, unless the control animals, which are fed normally, behave in a normal manner. In the present case, it has been shown that young chicks may be fed upon a normal dietary—which under better surroundings would suffice to produce a normal development—and yet suffer from severe forms of malnutrition.

We know well that there was no reason to expect the batches fed upon cooked "white" rice to behave in any manner other than they did, for it is definitely established that "white" rice is an inadequate diet in many respects.

It is however considered that it is unwise to draw any conclusions from the results obtained in dieting birds upon "red" rice. Although it is true that many of the birds which were fed in this manner, in this series of researches, did show a period during which arrested growth was a prominent feature; yet, many similar cases have been observed in the control chicks fed on normal food.

The photographs shown in this communication demonstrate that arrested growth may occur in normally fed chicks to almost as striking a degree as in the case recorded by Funk and Macallum.

The previous work of the authors just referred to, on growth in young chicks, therefore affords no satisfactory evidence of the existence of any accessory substances or, as Funk prefers to call them, "vitamines."

Needless to say, no attempt was made to carry out the proposed scheme of investigation into the effect of diet upon tumour growth in the young growing chick, nor will such an attempt be made in this Institute until it has been found possible to rear young birds indoors to a satisfactory standard of nutrition.

The observations reported in the present communication may, at first sight, seem incomplete without containing a more exhaustive enquiry into the cause which underlies the early failure of the young chickens. The advisability of carrying out such an investigation was, however, fully considered at the time, but it was felt that such a study would lead too far away from those problems which are, at the present time, engaging the activities of this Institute.

It is for this reason, therefore, that the results that were obtained have been communicated in their somewhat incomplete form.

Summary.

1. It has been found impossible to rear, to a satisfactory degree of development, young chicks kept under the artificial conditions prevailing in this Laboratory.

2. The age at which the chick is brought under the influence of these artificial conditions is apparently the chief determining factor in its subsequent development, other conditions being the same.

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Photograph 1. *Chick* 1 *A*, fed upon normal food, age 14 weeks, weight 500 g. At the time that this photograph was taken, this bird was at its prime. Nevertheless, the comb and eyelids were very pale and the bird was not strong. This chick remained at a body-weight of about 500 g. for nine weeks before it declined and died.

Photograph 2. *Chick* 207 *A*, age 12 weeks, weight 147 g. This bird was also fed upon normal food and belonged to the same batch as No. 1 *A*. It fed well, and seemed quite as strong as the bigger bird, despite its ill-developed appearance. The overgrowth of the wing feathers may be noted in this case. This chick remained at an almost constant body-weight for 80 days before it declined and died. Both photographs are on same scale.

VII. THE GROWTH OF RATS UPON ARTIFICIAL DIETS CONTAINING LACTOSE.

By JACK CECIL DRUMMOND.

From the Biochemical Laboratory of the Cancer Hospital Research Institute, London.

(*Received December 24th, 1915.*)

It has been known for some time that many natural food-stuffs contain substances which, although in many cases present in very small quantities, play an exceedingly important rôle in the processes of growth and nutrition.

A large amount of literature has been published during recent years which deals with these substances in one way or another. Particularly has interest been centred upon those substances of this class which if absent or deficient from the diet may induce a definitely pathological condition.

We are, however, in this paper concerned with another group of these so-called accessory substances, namely, those which are apparently indispensable to the diet of a growing animal if satisfactory growth is to occur.

Milk has been shown to be peculiarly rich in its content of growth-promoting accessory substances. Hopkins [1912] showed that a very small ration of milk added to a diet consisting of purified caseinogen, starch, lard and salts—a diet that by itself was inadequate to supply all the factors necessary for the growth of young rats—at once rendered it sufficient for this purpose. Stepp [1909, 1911, 1913] had previously shown that dietaries which had been extracted with alcohol were rendered incapable of sustaining the growth processes of young mice, but when the extracted substances were completely replaced normal growth was at once possible.

Stepp demonstrated that the replacement of many of the individual components of the alcoholic extracts, such as purified natural fats and lipoids, was not followed by any improvement in the animal's condition.

He therefore assumed that the factors responsible were in all probability some unknown members of the class of lipoid substances.

Feeding experiments were accordingly made by several workers, in which attempts were made to rear young growing rats to maturity upon fat-free diets.

Osborne and Mendel [1912] carried out an extensive series of experiments of this nature, using diets similar in composition to those utilised by them in their earlier researches [1911], but which had been subjected to extraction with ether. With such diets they found that it was possible to obtain satisfactory growth in young rats, over a period of time equal to that during which the rat normally shows growth, and concluded that growth is possible upon a fat-free diet.

Since their diets had not been extracted with hot alcohol, they naturally could not claim that they were lipoid-free, and they were even forced to admit that they had not been able to obtain growth in young mice when fed upon diets similar to those employed by Stepp. In spite of these facts, however, the views of the latter author were subjected to criticism.

That the active substance or substances may in some cases be removed from diets by treatment with fat solvents was shown when McCollum and Davis [1913] announced that an ether extract of butter added to a diet, inadequate in accessory substances, at once rendered the diet satisfactory. Osborne and Mendel [1913, 1, 2] also demonstrated that unsalted butter and butter fat possessed the same power.

It was then soon found that many naturally occurring oils and fats have this property associated with them, while, on the other hand, many other representatives of this class are devoid of any such activity.

McCollum and Davis, in the communication referred to above, also attempted to feed young rats upon a fat-free diet, composed of caseinogen, lactose, agar, dextrin and a suitable salt mixture. They too recorded results which apparently demonstrated that growth for a certain period of time was possible upon such a diet, but that, invariably, sooner or later, the animals fed upon it declined and died. This decline and death was not the result if the diet contained the ether extract of butter or egg yolk, for then the animals grew normally in every way.

While much work was being done upon the presence of the accessory substances occurring in company with many of the fats and oils, the possibility of other fractions of a normal dietary also containing other unknown substances possessing equally important properties was almost neglected.

Funk [1914, 1] and Funk and Macallum [1914] have, however, subjected Osborne and Mendel to criticism for the extensive use which the latter authors make of "protein-free milk." In this criticism it was pointed out that it was unsatisfactory to introduce into diets such an unknown factor as this complex mixture represents. McCollum and Davis [1915, 1] have also criticised the use of "protein-free milk," and have pointed out that the very differences, recorded by Osborne and Mendel themselves, in the nutritive value of natural "protein-free milk," as compared with an artificial product made from lactose and salts, were sufficient to render evident the existence of substances of an unknown nature, other than those usually carried by certain fats.

In this respect an observation made by Stepp is of interest. He favoured the belief that the substances present in milk, which play the part of accessory substances, are present not in the fat but rather in the skimmed milk fraction.

That there was any serious objection to the use of lactose in diets employed in feeding experiments has, however, not been considered fully until recently, when Hopkins and Neville [1913] repeated some of the earlier work of Osborne and Mendel. The former authors found that when purification of the various ingredients of an artificial dietary was carried to a further degree than usual, the animals declined at a much more rapid rate than when fed upon a similar diet which was less pure. In the preparation of the purified diets, they purified the caseinogen and starch by extraction with alcohol, and several times reprecipitated the lactose from aqueous solution by alcohol. Their results demonstrated clearly the importance of purifying the lactose employed in dietaries. Funk [1914, 1] also speaks of commercial lactose containing traces of nitrogen and therefore considers it is an unreliable ingredient to include in diets which are to be considered as "pure."

McCollum and Davis [1915, 1] have remarked that they appreciated the possible objection to the use of insufficiently pure lactose in diets. The samples of this sugar used by them, in their series of studies upon growth on fat-free foods, contained amounts of nitrogen varying from 0·02–0·034 %. In view of this fact they wisely refrained from any discussion as to the necessity of "vitamines," or hormones in the diets, until they had made an investigation of this point.

Sweet, Corson-White and Saxon [1915], in a research upon the influence of diet upon tumour growth in the rat, used diets similar to those employed by Osborne and Mendel, and they had occasion, from a consideration of their results, to believe that lactose does in some cases carry with it impurities

which may act as accessory substances to promote growth. They even explain some results obtained previously by van Alstyne and Beebe [1914], working upon the same subject, upon these grounds. The latter workers had fed rats upon two similar purified diets, one of which contained lactose, while the other did not. Investigations were made upon the effects of these two diets upon the growth of tumour grafts inoculated into the rats. From the results it was concluded that the carbohydrate of the diet, i.e. lactose, had had a beneficial effect upon the growth of the inoculated tumours in the rats. Sweet, Corson-White and Saxon now suggest that this result may be more correctly attributed to the presence of a growth-promoting accessory substance in the insufficiently pure lactose used in the diets.

In a recent paper Woglom [1915] has investigated the effect of lactose in diets upon the growth of tumours in rats and mice. The lactose was, however, added to their normal diet of bread and vegetables. From his results he concludes that lactose does not increase the receptivity of mice and rats for the transplantable carcinomata of their species and he proceeds to criticise the previous work of van Alstyne and Beebe. It must be remembered, however, that the latter authors added their lactose to a carbohydrate-free diet of caseinogen and lard, an important fact which Woglom has apparently passed over. The explanation of the results of van Alstyne and Beebe which has been given by Sweet, Corson-White and Saxon is, in all probability, the correct one. Any improvement in tumour growth in animals fed upon dietaries containing lactose is due, not to the lactose itself, but rather to the growth-promoting accessory substance which it so often carries, when insufficiently purified.

This work of Woglom therefore loses much of its value from the fact that he added the lactose to a diet already fully sufficient for normal growth. Consequently any action of the accessory substance, carried by the lactose he used, would fail to be apparent since both host and tumour-graft were already able to derive all the substances necessary for growth from the basal diet, without any addition of lactose.

Since the early spring of 1915, I have been making a large number of feeding experiments upon rats, with the object of obtaining sufficient experience in the technique to permit me to make an extensive examination of the effect of diet upon tumour growth. It was during these preliminary feeding trials that I was struck by the apparent effect of the purity of the lactose used upon the growth of the animal. Accordingly I made a few experiments to

ascertain the cause of this effect, and the results I have obtained are set forth in the present communication.

While the results were being compiled for publication, however, three papers appeared by McCollum and Davis [1915, 2, 3, 4], in which these indefatigable workers also record results consequent on the use of impure lactose in dietaries. They there put forward sufficient evidence to show that lactose, unless carefully purified, often does contain an accessory substance which helps to promote growth.

The identical conclusion to which the writer of the present paper had arrived, as a result of independent experiments, will now serve as a useful corroboration of the work of McCollum and Davis.

In view of the observations of Stepp, Hopkins, Funk and earlier workers, it has seemed a difficult matter to explain satisfactorily how Osborne and Mendel, and also McCollum and Davis, had been able to obtain a certain degree of growth in young rats fed upon fat-free diets composed of "purified" food-stuffs.

Osborne and Mendel have always been careful to utilise, throughout their extensive researches, proteins of a very high degree of purity, and yet they have gone to the other extreme in continuing to use in their diets "protein-free milk."

Reference has already been made to the criticisms which have been directed against the use of this complex substance, as a source of lactose and salts.

To determine whether any substance was contained in "protein-free milk" which would exert a growth promoting influence, two batches of young rats were taken and were fed upon the following diets.

Diet I.		*Diet II.*	
Caseinogen	18·0	Caseinogen	18·0
Wheaten starch	30·0	Wheaten starch	30·0
Lard	22·0	Lard	22·0
"Protein-free milk" ...	30·0	Extracted "protein-free milk"	30·0

All the ingredients of the diet other than the "protein-free milk" were carefully purified. The caseinogen was precipitated from machine-skimmed milk by acidification. It was then reprecipitated twice from dilute solution in sodium carbonate by the addition of acid, washed, pressed and dried.

The dried caseinogen was extracted with two changes of alcohol for six hours at 60°, and finally subjected to ether extraction for six hours.

McCollum and Davis [1915, 4] have now shown that caseinogen should be purified very carefully, owing to the fact that changes may occur in the protein molecule, if it is subjected to purification by strong measures, which may greatly lessen its nutritive value.

I have not found that the purification, to which I subjected the caseinogen used throughout these experiments, has rendered it unsuitable for the rats to any marked degree. The only two occasions on which I suspected that the caseinogen was unsatisfactory for the nutritive demands of the animals were when it had been extracted with hot alcohol whilst in a slightly moist condition. In these cases every rat upon the diets containing this caseinogen died within a short space of time. I have not yet investigated the matter carefully, but the result may be attributable to the caseinogen having undergone changes of an unknown nature.

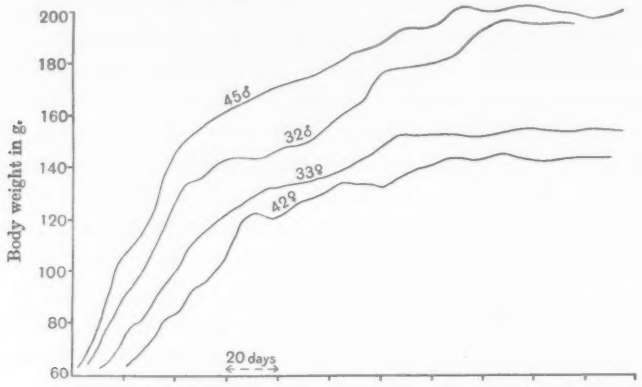


Chart I. Curve showing normal growth of young rats upon diet:

Milk powder	60
Lard	24
Starch	16

The wheat starch was also submitted to alcohol and ether extraction, while the lard was the purest obtainable.

The extracted "protein-free milk" was prepared by drying the product, obtained by following out the directions of Osborne and Mendel [1911], in an oven at 100° for six hours. The dried mass was then ground to a fine powder and extracted with alcohol at 60° C. and finally with ether.

To test whether the "protein-free milk" had been affected by the six hours' exposure to 100°, a third series of rats was placed upon a diet similar to Diet I, but which differed in containing the heated product.

Reference to Charts II, III, IV will show typical growth curves of rats fed upon these three diets, as compared with normal growth upon a milk powder diet, Chart I.

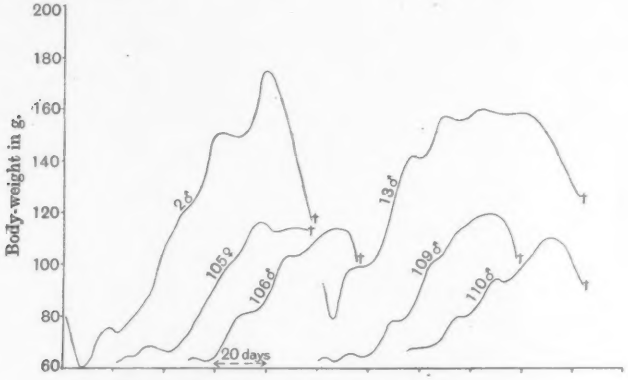


Chart II. Growth of young rats upon diet of:

Alcohol extracted caseinogen	18
Alcohol extracted starch	30
"Protein-free milk"	30
Lard	22

Growth was obtained upon the diet containing "protein-free milk" and also to about the same degree upon that containing the heated product. The growth curves in Chart II are in every respect similar to those published by Osborne and Mendel, using similar diets. Approximately normal growth

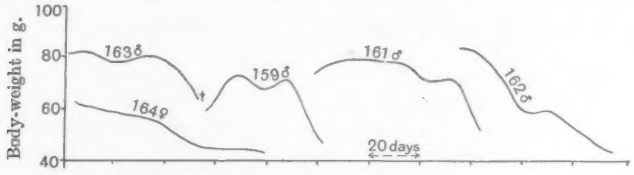


Chart III. Failure to grow of rats fed upon diet:

Alcohol extracted caseinogen	18·0
Alcohol extracted starch	30·0
Heated and alcohol extracted "protein-free milk" ...	30·0
Lard	22·0

occurs for periods of from sixty to eighty days, and is followed by a cessation of growth and a rapid decline. This cessation and rapid decline may be prevented, and the animal may continue to grow normally, if at the critical point one of the fats containing accessory substances is added to the diet.

The rats fed upon the diet containing heated and extracted "protein-free

milk," however, showed no signs of growth whatever (Chart III). Slow decline characterised the period during which they were fed upon the diet, and this decline usually terminated in death after some forty days or so.

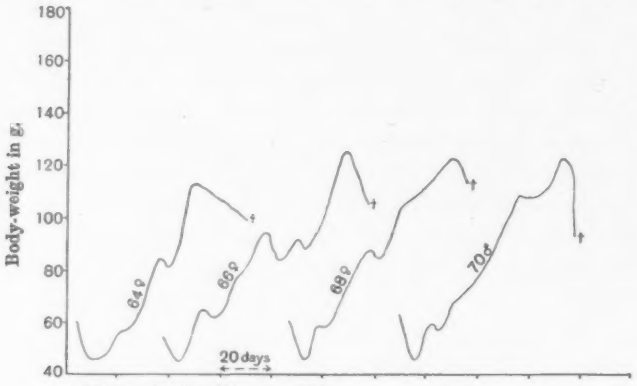


Chart IV. Growth upon diet:

Extracted caseinogen	18·0
Extracted starch	30·0
Lard	22·0
Heated "protein-free milk"	30·0

Obviously, some substances had been removed from the "protein-free milk," by the alcohol extraction, that were indispensable.

Similar results, I find, are recorded by MacArthur and Luckett [1915] who, investigating the indispensability of lipins in the diet, also subjected their "protein-free milk" to a similar extraction in order to remove from it all traces of this class of compound.

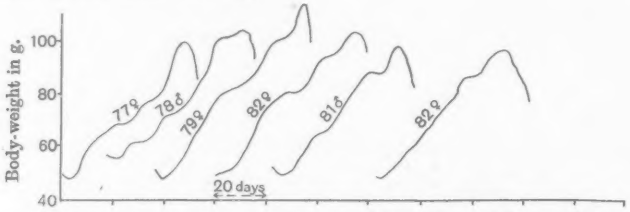


Chart V. Slow growth, and subsequent failure of rats fed upon diet:

Alcohol extracted caseinogen	18·0
Alcohol extracted dextrin	56·0
Alcohol extracted agar	2·3
Salt mixture *B*	3·7
Lactose	20·0

Experiments of a similar nature were then made, using a fat-free diet which has been used extensively by McCollum and Davis [1915, 1].

Diet IV.

Caseinogen	18·0
Dextrin	56·0
Lactose	20·0
Agar	2·3
Salt mixture	3·7

The salt mixture employed was one used with satisfactory results by these authors, and is composed of:

NaCl	0·173 g.
$MgSO_4$ (anhyd.)	0·266 „
$NaH_2PO_4H_2O$	0·347 „
K_2HPO_4	0·954 „
$CaH_4(PO_4)_2$ $2H_2O$	0·540 „
Calcium lactate	1·300 „
Ferric lactate	0·118 „

Iodine was supplied in the drinking water (distilled) once weekly.

Upon this diet it was found that the stronger of the rats grew at a satisfactory rate for a period of about 50 days (Chart V). They did not reach as high a maximum weight as did the series fed upon Diet I, i.e. a diet in which the lactose was in a cruder form,—and having grown as indicated, they soon rapidly declined and died. Again in this case, the decline and death could be prevented by the prompt addition to the diet of a fat-soluble accessory substance.

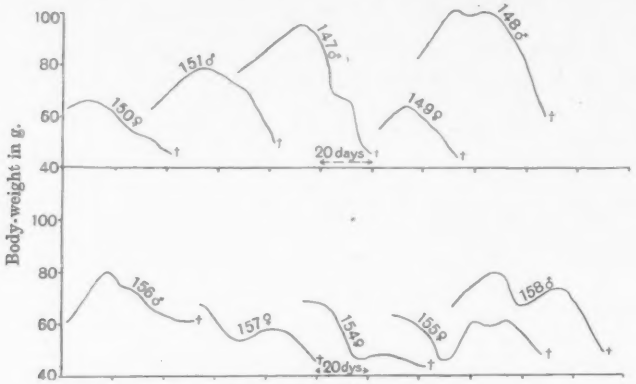
As all the ingredients of this diet, other than the lactose, had been subjected to careful purification, the results obtained showed that it would be advisable to investigate the effect of purifying the lactose.

The lactose used throughout these experiments was bought from reliable English firms as "pure," but notwithstanding this it usually gave, on analysis, evidence of the presence of nitrogen. The average figure obtained from analyses made on several samples of the sugar gave the percentage content of nitrogen as 0·024 %.

Samples of lactose as supplied under the description of "pure" and "purest" vary largely in their nitrogen content. I have had samples which were practically nitrogen free, and I can now attribute some of my failures to obtain growth upon such diets as Diet IV to this cause. The lactose was purified as Hopkins and Neville have described, namely, by precipitation from aqueous solution by means of alcohol.

A ration similar to Diet IV was then made up, containing the once recrystallised lactose instead of the cruder product.

The curves, showing the slight growth of rats fed upon this Diet V (Chart VI), demonstrate fully that the one purification of the lactose by recrystallisation had removed the majority of the growth promoting factor.



Charts VI and VII.

Chart VI. Slight growth, followed by rapid decline, upon diet:

Alcohol extracted caseinogen	18·0
Alcohol extracted starch	56·0
Alcohol extracted agar	2·3
Salt mixture B	3·7
Recrystallised lactose	20·0

Chart VII. Practically no growth upon diet:

Alcohol extracted caseinogen	18·0
Alcohol extracted starch	46·0
Alcohol extracted agar	2·3
Salt mixture B	3·7
Recrystallised lactose	20·0
Lard	10·0

A point of interest may be noted here. Using a diet similar to V, but containing some 10 % of lard, I was unable to obtain, in a large series of rats, more than one or two cases showing any growth whatever. The majority of the animals in this group slowly declined from the outset (Chart VII).

I cannot say, as yet, whether this result was purely accidental, or whether it had its origin in the inclusion of the lard in the diet. The possibility of the latter view being correct is not altogether out of the question, as parallel cases do exist. For instance, it is known that the quantity of carbohydrate in a diet plays a very important part in governing the utilisation of the

accessory substance which prevents beri-beri [Funk, 1914, 2; Braddon and Cooper, 1914].

Another diet was then tried which contained a much larger percentage of the unpurified lactose.

Diet VI.

Caseinogen	18·0
Lactose	56·0
Dextrin	20·0
Agar	2·3
Salt mixture	3·7

It was expected, from the greater percentage of lactose in this diet, that growth would be still more successfully promoted, and experimentally this expectation was realised (Chart VIII).

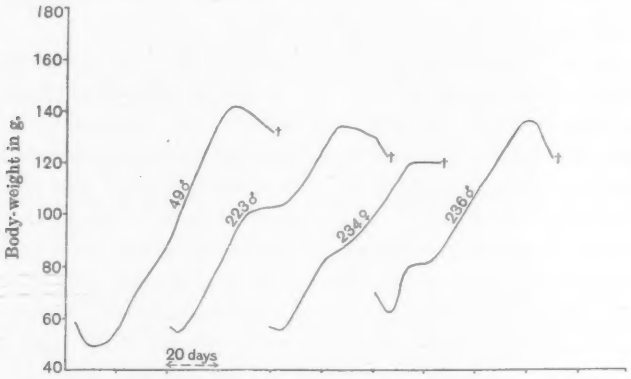


Chart VIII. Growth and early failure of young rats upon diet:

Extracted caseinogen	18·0
Extracted lactose	56·0
Extracted dextrin	20·0
Extracted agar	2·3
Salt mixture B	3·7

The animals survived, as a whole, longer, grew to a slightly greater maximum body-weight, and the subsequent decline did not occur at such an early date as in the cases of the rats fed upon a similar diet, which, however, contained only 20 % of lactose.

McCollum and Davis have just pointed out [1915, 3] that, if the lactose in a diet similar to this is entirely replaced by dextrin, no growth whatever is obtained, whilst Lander [1915] found no growth in young rats fed upon purified diets containing cane-sugar instead of lactose.

In another series of feeding experiments, lactose was used which had been twice recrystallised in the manner already indicated. No growth at all resulted, and the rats rapidly declined and died [Chart IX].

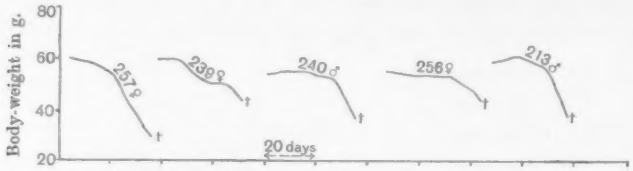


Chart IX. Rats fed upon diet:

Caseinogen	18·0
Dextrin	56·0
Agar	2·3
Salt mixture	3·7
Twice recrystallised lactose	20·0

A preliminary attempt was made to concentrate the active substance to some extent in the following manner.

The mother-liquors from a series of recrystallisations of lactose were concentrated down to dryness at 40° under diminished pressure. The resulting impure lactose was then recrystallised once from aqueous solution by means of alcohol. The mother-liquor from this recrystallisation was evaporated as before to dryness, and the product obtained was utilised in a diet, as the source of lactose, to the extent of 20 %. The effects of this concentration of the accessory substance upon the growth were not as marked as were expected. Chart X illustrates the type of growth curve of rats fed upon this ration. The maximum body-weight attained was slightly greater than that reached upon Diet IV, but no postponement of the rapid decline was observable. A further attempt is being made in this direction.

One cannot help being much impressed by the apparent need of the growing animal organism for more than one type of accessory substance. The one factor that has been studied in this paper is itself useless to make good the inadequacy of a pure diet so that complete growth can result. This can only occur when the diet also contains one of the fat-soluble accessory substances.

McCollum and Davis, in the recent papers referred to, have shown that many food-stuffs carry a water-soluble accessory factor that is of quite as great importance as those usually associated with many fats.

Thus wheat-embryo, egg-yolk and other substances are shown to contain water-soluble substances which may now be classified as accessory substances. Oseki [1914] had already shown that the accessory substance present in rye

is soluble in water, while Funk and Macallum [1914] have suggested the likelihood that two classes of accessory substances—called by Funk "vitamines"—exist, one occurring in water-containing food-stuffs, such as fruit.

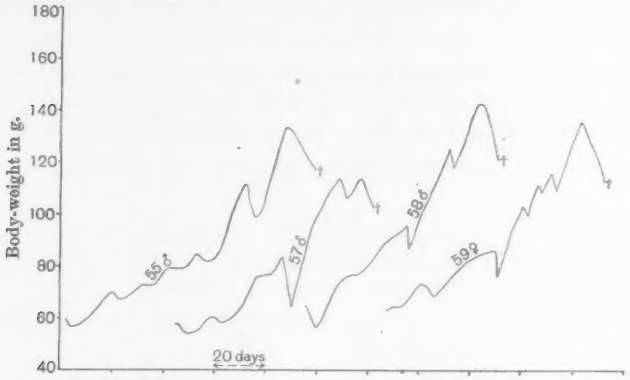


Chart X. Growth upon diet:

Caseinogen	18·0
Dextrin	56·0
Agar	2·3
Salt mixture B	3·7
Lactose + concentrated impurities	20·0

whilst the other is generally found in the company of fats such as butter and cod-liver oil.

Conclusions.

It is apparent, from a consideration of the results set forth in the present communication, that lactose may contain effective amounts of a growth promoting factor, soluble in alcohol and stable on exposure to 100° for six hours.

These results are in entire agreement with those recently published by McCollum and Davis, and both sets of conclusions show that the suspicion with which other workers, to whom reference has been made, have regarded the inclusion of insufficiently pure lactose in experimental diets was well grounded.

It is now established conclusively that the views, held for some time past by Stepp, Hopkins, Funk and other workers, are correct, and that growth is impossible in the young animal when the diet supplied to it is deficient in certain indispensable accessory substances.

Our knowledge of the action of these unknown factors is so slight, that we shall be forced to reconsider many of the conclusions which have been drawn from the results of feeding experiments, involving the use of diets which we now know were not pure.

Much extremely valuable work has been done in this field, but the fact that many of the diets employed contained lactose, in an impure form, will make one await with interest the results which will be obtained when these experiments are repeated with diets containing a purer carbohydrate.

In this light, we may now regard many of the failures to obtain growth in young rats upon artificial food rations, which have been recorded by numerous workers, as evidence that they were truly dealing with diets of purified substances, and not as being due to faulty technique as has been suggested by some workers, whose own methods are now open to criticism.

Summary.

1. Lactose, prepared from milk, may contain traces of an impurity which acts as a growth-promoting accessory substance.

2. This substance is soluble in water and alcohol, and is not destroyed by exposure to 100° for six hours.

3. Before complete growth can occur in a young animal, the diet, besides being adequate as regards its proteins, carbohydrates, fats and salts, must contain both fat-soluble and water-soluble accessory substances.

4. No growth is possible in the animals fed upon a purified diet which is entirely deficient in these accessory substances.

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VIII. NOTE ON THE ISOLATION OF METHYLGUANIDINE BY THE SILVER METHOD.

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In the course of some experiments which were carried out in relation to the "diazo reaction" of the urine of typhoid patients, it was found desirable to submit the urine to Kutscher's process for the isolation of bases. The process, as is well known, consists in fractional precipitation by means of silver, first in acid and subsequently in alkaline solution. Treatment of the urine by this means resulted in the isolation of an unexpectedly large amount of methylguanidine. Thus, while previous workers, Kutscher and Lohmann [1906], Engeland [1908] and Achelis [1906], had obtained varying amounts of methylguanidine which corresponded to an average yield of 0·01 g. of base per litre, the average amount obtained in the experiments under consideration was approximately 0·06 g. per litre, and in one case 0·17 g. per litre was isolated.

It was at first thought that this result was of some pathological significance, but in order to confirm the earlier results recorded in the literature, a quantity of normal urine was submitted to a process of fractionation similar in all respects to that carried out upon the typhoid urine. The result was somewhat unexpected; an amount of methylguanidine being obtained approximately corresponding to 0·07 g. per litre.

From this result it appeared either that the amount of methylguanidine present in normal urine had been underestimated, owing to incomplete isolation, or that under the conditions of fractionation as carried out in the above experiments methylguanidine had been produced from some other substance present in the urine.

It is at once evident that the last hypothesis is by no means improbable. Methylguanidine, as is well known, is readily obtainable from creatinine (or creatine) by oxidation, either by boiling with mercuric oxide in acid solution, as first shown by Dessaignes [1854], or according to Neubauer [1861] by permanganate. It seemed therefore by no means unlikely that in the presence of silver oxide produced in the fractionation, a certain amount of the creatinine of the urine would be oxidised in a similar manner giving rise to methylguanidine. The larger yield of the latter base obtained in the present instance would readily be explained by the fact that in order to avoid precipitation of creatinine as far as possible, a large excess of baryta solution was employed and the precipitated solution allowed to stand for some considerable time in accordance with the process employed by Engeland [1908]. Experiments were therefore carried out in which pure creatinine was submitted to the conditions obtaining in the process of fractionation. To the solution of pure creatinine, silver nitrate was added until such an excess was present that on addition of baryta an immediate brown precipitate (which rapidly became black) was produced. Warm saturated baryta solution was then added in excess. After suitable treatment methylguanidine was readily isolated as a crystalline picrate. Further, it was shown that, according to the conditions under which the fractionation was carried out, larger or smaller amounts of methylguanidine were produced. Thus, working as rapidly as possible at a low temperature, only a very small amount of methylguanidine was obtained, while if the solutions used were warm, larger quantities were produced. By heating creatinine with silver nitrate and baryta in excess on the water bath for an hour or so, a nearly quantitative yield of methylguanidine can be obtained, this method indeed being perhaps the most suitable for the preparation of the base in quantity.

In view of the foregoing results, it would appear that the statements found in the literature with regard to the occurrence of methylguanidine in urine, muscle, etc. must be looked upon with some distrust, at any rate in those cases where the base has been isolated by the Kutscher process.

According to Kutscher [1905] and Gulewitsch [1906] commercial meat extracts contain large amounts of methylguanidine (0·38 % of the nitrate according to Gulewitsch), but since the base was isolated as described above, and warm baryta solution was employed, the figures obtained were at least considerably too high. Krimberg [1906] again obtained methylguanidine from fresh horse meat by the silver fractionation, whereas Brieger [1886] using another method was only able to obtain it from horse meat after

putrefaction. Smorodinzew [1913] obtained 0·083 % from fresh horse meat and also [1912] found the base in liver. It is to be noticed however that by a preliminary fractionation of the phosphotungstic precipitate from extract of horse meat he obtained two fractions, one of which contained creatine, the other being creatine-free. From the former fraction only could methylguanidine be obtained.

The amounts which have been isolated from normal urine are in all cases small. It is perhaps significant that Achelis [1906] failed to isolate any methylguanidine from normal urine until the fractionation had been repeated once or twice (*i.e.* only after somewhat prolonged treatment with silver and baryta). On the other hand, Kutscher and Lohmann [1906] obtained small amounts by another process of fractionation. The statement by Koch [1912] that a large amount of methylguanidine (1·9 g. of the gold salt per litre) occurs in the urine of parathyroidectomised dogs appears to be beyond criticism since the method of isolation employed did not involve the use of silver and baryta.

Heyde [1911] has also stated that the urine of animals killed by anaphylactic shock contains a large amount of methylguanidine, but no details of the method employed for its isolation are given.

Experimental.

Fractionation of Urine. (A.)

To 1420 cc. of urine, which in this case was that of a typhoid patient, were added 380 cc. of a 20 % aqueous solution of normal lead acetate. The precipitate was filtered off and the filtrate concentrated to about 100 cc. The concentrated solution was neutralised and then completely precipitated by alternate addition of a hot saturated aqueous solution of mercuric chloride and a saturated solution of sodium acetate until a portion of the filtrate no longer gave more than an opalescence on further addition of either reagent. The precipitate was filtered off, suspended in dilute hydrochloric acid solution and digested on the water bath until the reaction remained permanently acid to Congo red. The insoluble portion was removed by filtration and discarded. The acid filtrate was decomposed by sulphuretted hydrogen, the mercuric sulphide filtered off, the filtrate freed from sulphuretted hydrogen by a current of air, neutralised with caustic soda and evaporated to dryness *in vacuo*. The residue was completely extracted with hot methyl alcohol, the solvent distilled off under reduced pressure and the residue taken up in

a little water. The aqueous solution was made just acid to Congo red with dilute nitric acid and a 20 % aqueous solution of silver nitrate added until precipitation was complete. This precipitate (containing the purine bases) was filtered off and set aside.

To the filtrate (the volume of which was 150 cc.) was then added silver nitrate until the solution gave an immediate brown precipitate with baryta solution. Warm saturated baryta solution was then added in excess and some additional solid baryta also stirred into the solution, in order to ensure the decomposition of the creatinine silver compound. After 24 hours the precipitate was filtered off, re-dissolved by addition of dilute nitric acid, filtered from insoluble material, and again precipitated with excess of baryta solution. After standing, the precipitate was separated by centrifuging, washed by suspending in water and again centrifuging and finally dissolved in dilute nitric acid and filtered. The clear acid filtrate was then treated with dilute aqueous ammonia until a little of the filtered solution gave no precipitate with ammoniacal silver nitrate. The yellowish white precipitate was filtered off and worked up in the usual way.

The filtrate was completely precipitated by addition of saturated baryta solution. The precipitate was filtered off, washed well with water, suspended in water and decomposed by sulphuretted hydrogen. The filtrate was evaporated to small bulk and on treatment with hot saturated picric acid solution gave a crystalline picrate which on recrystallisation yielded 1·05 g. of pure methylguanidine picrate, melting at 201–202°. The identity of the base was established by direct comparison of the picrate with methylguanidine picrate obtained from another source and by direct conversion into the crystalline nitrate.

In another experiment in which 7·7 litres of typhoid urine were fractionated under similar conditions except that the treatment with silver and baryta was less prolonged, a final yield of 2·05 g. of methylguanidine picrate was obtained.

Fractionation of urine. (B.)

4,600 cc. of normal urine were fractionated exactly as described above. The yield of pure methylguanidine picrate was in this case 1·3 g.

Conversion of creatinine into methylguanidine.

1·8 g. of creatinine obtained by decomposition of the creatinine zinc salt ($Kr_2SO_4ZnSO_48H_2O$) [Folin and Blanck, 1910] was dissolved in water and

the solid made acid to Congo red with dilute nitric acid. A 20 % aqueous solution of silver nitrate was then added until a brown precipitate was immediately obtained on treating a drop of the solution with baryta. Warm saturated baryta solution was then added in excess and the mixture allowed to stand over night. The precipitate was then filtered off, washed, redissolved in dilute nitric acid and the process repeated. The precipitate so obtained was dissolved in dilute nitric acid and fractionated. Dilute aqueous ammonia was added until no precipitate was obtained on addition of ammoniacal silver nitrate to the filtered solution. The precipitate so formed was filtered off and the filtrate precipitated by addition of bartya solution in excess. The precipitate was suspended in water decomposed by sulphuretted hydrogen, freed from traces of barium and concentrated. On addition of hot saturated picric acid to the concentrated solution, 0·6 g. of a crystalline picrate melting at 201–202° was obtained which was identified as methylguanidine picrate.

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IX. THE STABILITY OF PANCREATIC LIPASE.

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(*Received February 3rd, 1916.*)

For some years glycerol extracts of pancreas have been extensively used in this laboratory by Rosenheim, Shaw-Mackenzie and others. The lipase contained in the extracts has been the centre of interest, but the presence of other enzymes and the bearing this may have on the behaviour of lipase have not been overlooked. The most important of these enzymes, in this respect, is trypsin, which has often been shown to be present in these glycerol extracts.

Conflicting statements with regard to the action of trypsin on lipase appear in the literature. Experiments with pancreatic juice carried out by Terroine [1910] and by Mellanby and Woolley [1914] show that lipase rapidly disappears in presence of trypsin. Mellanby and Woolley state further that glycerol extracts of pancreas contain trypsinogen and enterokinase, and that activation is inhibited by the glycerol; but that dilution removes this inhibitory influence and trypsin is formed which destroys the lipase present in the extract. Shaw-Mackenzie [1915], on the other hand, claims that trypsin is present in the concentrated extract, and Tsuji [1915] has shown that dilution not only does not abolish, but may even increase, the lipoclastic activity.

These contradictory statements leave us in a state of uncertainty as to the stability of the lipase in glycerol extracts of pancreas. All the extracts used in this laboratory have been strongly lipoclastic and those tested have been found to be proteoclastic. An extract has been employed for class work for the last two years and has suffered no deterioration in its fat-splitting power during that time. Dr Shaw-Mackenzie has kindly supplied me with

notes showing that some of the extracts prepared by Rosenheim and himself were active more than two years after they were made. As several of these extracts have been kept, I have taken the opportunity to investigate further the stability of the lipase.

Experimental.

Preparation of the extracts.

All the extracts were prepared in the same way. Fresh pig's pancreas was freed from fat, minced and weighed. It was then allowed to stand for 24 hours with glycerol, 2 cc. of glycerol being used for 1 g. of pancreas. The extract was strained off through two layers of muslin, and kept in a stoppered bottle. Just before use one part of this concentrated extract was diluted with two parts of water.

Estimation of lipoclastic activity.

The method employed was that used by Rosenheim and Shaw-Mackenzie [1910]. 5 cc. of an olive oil emulsion were incubated with 3 cc. of the dilute extract at 37° for 18–24 hours and the fatty acids estimated by titration with N/10 NaOH, alcohol being added to facilitate titration and phenolphthalein being the indicator. The estimations were done in duplicate and the means of the results were corrected for the original acidity of the mixture of emulsion and extract. The details of the method have recently been very fully described by Umeda [1915]. I find, with him, that the quality of the oil is of importance. The length of time during which the mixture of extract and emulsion is shaken has also to be considered. Bradley [1909] has investigated the effect of shaking emulsions, and his results show that, if an emulsion be shaken too long, lipoclasis is lessened. He suggests in explanation that aëration destroys the enzyme or makes it difficult for the enzyme to reach and act upon the oil. While this may be the explanation in many cases, some of my experiments show that, in shaking the mixture of extract and emulsion, a difference in time of only ten seconds causes the oil to separate out, thus producing a condition which should retard lipoclasis. In the following experiment it will be seen that shaking for ten seconds gave a much better result than shaking for twenty seconds.

Exp. 1. Flasks containing 3 cc. of dilute extract and 5 cc. of oil emulsion were shaken for 5, 10, and 20 seconds, respectively, and put into the incubator at 37° for 22 hours. The mixture, before incubation, required 2 cc. of

N/10 NaOH to neutralise it. This number was subtracted from those obtained after incubation, and the corrected numbers are given.

Time of shaking, secs.	N/10 NaOH cc.
5	32·8
10	35·2
20	20·4

The oil had separated out in the last flask only. With an oil resistant to lipase this difference is not appreciable as the numbers obtained are all small. This may be due to the fact, pointed out by Umeda [1915], that the oil contains an impurity which resists the action of lipase even when the oil is finely emulsified. Such oils, too, often form thin emulsions, and when mixed with the extract they separate out in the incubator, or, more rarely, on being shaken for a short time. The inappreciable differences produced by shaking, and the low numbers obtained with these oils, are evident on comparing the following with the preceding experiment, the extract used being the same in both cases.

Exp. 2. Pairs of flasks containing 3 cc. of dilute extract and 5 cc. of oil emulsion were shaken for 5, 10, and 20 seconds, respectively, and put into the incubator at 37° for 18 hours. The mixture, before incubation, required 1·1 cc. N/10 NaOH to neutralise it. The mean of the titration numbers for each pair, after incubation, was taken, and these means were corrected for the original acidity. The final numbers are given.

Time of shaking, secs.	N/10 NaOH cc.
5	3·3
10	3·4
20	3·1

In order that differences due to shaking might not vitiate the experiments, the mixture of extract and emulsion was shaken slowly ten times in all the subsequent work.

Estimation of tryptic activity.

The tryptic activity was measured by Sörensen's [1908] formaldehyde method, decinormal sodium hydrate being used for titration.

The following was the usual procedure:

3 cc. of dilute pancreatic extract and 10 cc. of a 2 % solution of caseinogen in 0·4 % sodium carbonate were put into each of three flasks. The amino-acids were at once estimated in one of these by the addition of 10 cc. of neutral formalin and subsequent titration with N/10 NaOH. The other two

flasks, after the addition of 0·5 cc. of toluene to each, were put into the incubator at 37° for 18–24 hours. The amino-acids were then estimated in the same way. The mean of the numbers obtained for these two was taken, and from it was subtracted the number obtained for the control flask. The result was a measure of the proteoclastic activity.

The activity of old extracts.

Ten extracts which had been prepared at periods varying from nearly six to three years ago were examined, and their lipoclastic and proteoclastic powers compared with those of an extract three months old (No. 11 in the series) and known to be active. The extracts were diluted before use for the lipoclastic but not for the tryptic determinations, and the experiments were carried out in the manner already described, lipoclasis going on at 37° for 18 hours, and proteoclasis for 24 hours. Each experiment was performed in duplicate and the mean of the results corrected for the original acidity of the mixture of extract and substrate. For the numbers illustrating the lipoclastic activity (after 18 hours at 37°) of the extracts soon after preparation I am indebted to Dr Shaw-Mackenzie. The proteoclastic activity at that time was not recorded. The numbers are collected together in the following table.

	Lipoclastic activity in cc. N/10 NaOH		Time interval between (I) and (II)		Tryptic activity in cc. N/10 NaOH
No.	(I)	(II)	yrs.	mths.	
1	14·8	31·2	5	8	6·3
2	23·6	26·6	5	7	4·9
3	28·9	20·7	5	4	3·9
4	16·8	15·9	5	4	3·9
5	12·6	5·2	5	4	2·6
6	14·1	24·1	5	3	3·7
7	28·1	26·5	5	2	5·4
8	13·3	26·6	4	10	4·3
9	39·2	15·5	3	11	3·8
10	25·8	27·6	3	4	5·4
11	—	30·0	—	—	7·4

(I) shows the original and (II) the present lipoclastic activity. The numbers in (I) are not strictly comparable with each other as the estimations were not all made with the same oil; those in (II) are comparable with each other, having all been obtained under the same conditions, but are not strictly comparable with those in (I). It is evident, however, that all the extracts are still lipoclastic and, except No. 5, compare favourably with the recent preparation No. 11. No. 5 is a thin extract and I suspected that it had been

diluted, but Dr Shaw-Mackenzie's notes showed that it was made in the usual way, and there is no reason to believe that it has since been tampered with. It is evident, too, from the last column, that all these extracts are tryptic. Mellanby and Woolley [1914] state that glycerol extracts of pancreas are tryptic only on dilution. Shaw-Mackenzie [1915] has shown, by the serum plate method, that the *concentrated* extract is tryptic. I am able to confirm this, for, with the serum plate method, all our concentrated extracts are tryptic. Concentrated glycerol extracts of pancreas, therefore, can be kept for a considerable time and still retain lipoclastic and proteoclastic activity, the glycerol apparently inhibiting the destructive action of trypsin on lipase.

The stability of dilute extracts.

For most of our work the concentrated extract was diluted with twice its volume of water shortly before use. To what extent this dilute extract retains lipoclastic and proteoclastic activity after it has been kept for some time, has been the subject of further investigation.

The following preparations were made:

A.	Concentrated glycerol extract ...	67 cc.
	Distilled water	133 „
B.	Concentrated glycerol extract ...	67 „
	1 % solution of formaldehyde ...	10 „
	Distilled water	123 „

(= 1 in 2000 formaldehyde)

The lipoclastic and proteoclastic activities of these preparations were at once examined in the usual way. The flasks containing them were corked and left at room temperature for 5 days when a second examination was made. To A, from which 18 cc. in all had been removed, 2 cc. of toluene were added. Both flasks were corked and left at room temperature. Forty-six days after the date of preparation they were again examined. In all the examinations lipoclasis and proteoclasis went on for 24 hours in the incubator at 37°. The results are here given.

		A	B
Lipoclastic activity in cc. N/10 NaOH	at once	30·9	29·8
	after 5 days	30·1	29·1
	after 46 days	29·3	25·1
Tryptic activity in cc. N/10 NaOH	at once	5·1	4·8
	after 5 days	4·2	4·2
	after 46 days	3·2	3·3

The tryptic power of both had diminished to some extent, but in spite of the presence of trypsin both preparations were still strongly lipoclastic, the activity of the toluene preparation being practically unimpaired while that of the formaldehyde preparation was slightly diminished. The preparations gave no odour of putrefaction.

Even with greater strengths of formaldehyde the extracts retain both lipoclastic and proteoclastic activity.

The following preparations were made:

No.	Concentrated extract cc.	Distilled water cc.	1 % formaldehyde solution cc.	Toluene cc.
I	100	200	—	—
II	100	200	—	4
III	100	170	30	—
IV	100	140	60	—

The strength of formaldehyde in III was 1 in 1000 and in IV 1 in 500.

The lipoclastic and proteoclastic activities were examined in the usual way, incubation going on for 22·5 hours at 37°. The flasks containing the preparations were corked and left at room temperature. After 42 days No. I contained a mould (*Penicillium glaucum*) but there was no odour of any kind, No. II gave the odour of toluene, and Nos. III and IV had an oily but not putrid odour.

In every case, as also in the last experiment, a precipitate had separated out and risen to the top. The mixture was therefore always shaken before samples were removed for examination.

The activities just after preparation and 42 days later are given.

		I	II	III	IV
Lipoclastic activity in cc. N/10 NaOH	at once	23·4	23·4	22·7	23·7
	after 42 days	23·4	28·9	16·4	13·1
Tryptic activity in cc. N/10 NaOH	at once	5·5	5·5	5·3	4·9
	after 42 days	5·1	5·3	5·0	4·6

All were still lipoclastic and proteoclastic. The proteoclastic activity showed no appreciable change. The lipoclastic activity was diminished in the extracts containing formaldehyde but gave a higher number in that containing toluene. The activity of the extract without antiseptic was in no way impaired. It is interesting to note that formaldehyde, in the strengths used, did not destroy the lipase.

Thus it appears that in these dilute extracts trypsin and lipase can exist together without destruction of the latter, the extracts containing enough glycerol to protect the lipase.

Conclusions.

1. Glycerol extracts of pancreas retain their lipoclastic and proteoclastic activity even after more than five years.

2. Dilute extracts (1 in 3), kept with or without antiseptics for six weeks, show both lipoclastic and proteoclastic activity.

3. Trypsin and lipase are present together in concentrated and dilute glycerol extracts, the glycerol apparently inhibiting the destructive action of trypsin on lipase.

I wish to thank Dr Rosenheim for suggesting to me the subject of this research and for much kind assistance.

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X. THE ANALYSIS OF PROTEINS. I. THE ESTIMATION OF ARGININE BY DECOMPOSITION WITH ALKALI.

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In the procedure suggested by van Slyke [1911] for the analysis of proteins, the arginine, which is precipitated together with the histidine, lysine and cystine by phosphotungstic acid, is estimated by boiling the solution containing these four compounds with 50 % caustic potash for six hours. Under these conditions the guanidine grouping of the arginine is decomposed with the formation of ammonia, which is collected in excess of standard acid and determined by titration. Twice the amount of the nitrogen as ammonia corresponds to the amount of arginine according to the equation:

$$H_2N\,(NH)\,C\cdot NH\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH\,(NH_2)\cdot COOH + 2H_2O$$
$$= 2NH_3 + CO_2 + H_2N\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH\,(NH_2)\cdot COOH.$$

In carrying out analyses of proteins by this method difficulty has been experienced in obtaining data for the amount of arginine. Numerous determinations have been lost through the action of the strong caustic alkali upon the glass vessels and duplicate analyses have not agreed with one another. Further, results obtained by the substitution of a copper vessel in which the alkaline decomposition was carried out were generally higher than those obtained with glass vessels. It became necessary therefore to enquire more closely into the decomposition of arginine and the other compounds by caustic alkali.

Previous workers have not described any detailed experiments upon the action of alkali upon arginine. Schulze and Likiernik [1891] described the

formation of urea by boiling arginine with saturated baryta water and of ammonia and carbon dioxide by heating arginine under pressure with baryta water. Osborne, Leavenworth and Brautlecht [1908] found that arginine was slowly decomposed by distillation with 0·1 N sodium hydroxide, and van Slyke [1911] makes no other statement than that arginine is completely decomposed by boiling with 50 % caustic potash (12·5 g. KOH to 25 cc. solution) for six hours.

Cystine, as is well known and as was pointed out by van Slyke, is decomposed by boiling with alkali, but its amount in proteins other than the keratins is usually so small that the amount of ammonia evolved from it is negligible: in the case of the keratins van Slyke gives a correction.

Lysine is a diamino acid and is stable to alkali.

Histidine contains a glyoxaline ring in its molecule and it is possible that this ring is broken down with liberation of ammonia by the drastic treatment with caustic alkali.

(1) *Action of Caustic Alkali upon Arginine.*

The arginine used in these experiments was prepared by the usual method from gelatin and isolated as carbonate. This salt, instead of the nitrate, was chosen so as to avoid any possibility of the action of nitrates upon the decomposition, but it is more difficult to obtain in a pure state. The recrystallised preparation consisted partly of carbonate and partly of free base as shown by its nitrogen content of 26·2 %; the free base contains 32·0 % and the carbonate 23·7 %.

Solutions of this preparation of about 0·1 % concentration were gently boiled with caustic soda of various strengths for periods of 6 to 11 hours either in a glass flask of about 200 cc. capacity or in a copper flask of about 400 cc. capacity; to the flask was attached a condenser and to the condenser a U-tube of the pattern devised by Folin. The experiments were carried out exactly as described by van Slyke.

The results were as follows:

(a) With the copper flask.

Concentration of NaOH %	Time of boiling hours	NH_3 evolved in cc. 0·1 N	One-half total nitrogen in solution in cc. 0·1 N
10	6	9·2	11·4
50	6	27·4	28·5
20	6	10·7	11·4
20	8	8·3	8·7
20	8	7·6	8·0

(*b*) *With a glass flask.*

	Concentration of NaOH %	Time of boiling hours	NH_3 evolved in cc. 0·1 N	One-half total nitrogen in solution in cc. 0·1 N
I	4	6	17·2	28·5
	10	6	8·8	11·4
	20	6	10·6	11·4
II	20	8	7·8	8·0
	50	6	7·3	8·0
	20	6	7·3	8·0
	20	6	7·3	8·0
	20	6	7·3	8·0
III	20	7	3·2	2·8
	20	7	2·5	2·8
	20	6·5	3·2	2·8
	20	6·5	2·7	2·8

The experiments were performed in three groups with different quantities of total nitrogen in solution. In the first group it was found at once that 4 and 10 % caustic soda did not give an amount of ammonia corresponding to half the amount of arginine nitrogen in solution, and that 20 % gave nearly the correct amount. In the second group confirmation of the previous result was obtained; the same result was given when 50 % caustic soda was used. Further confirmation is given by the experiments in the third group. In all experiments an amount of ammonia corresponding to half the total was never obtained. This was probably due to impurity in the preparation of arginine, which was not purified by conversion into the nitrate or the copper nitrate double salt. The experiments show that the estimation of arginine can be effected by boiling with 20 % caustic soda in place of 50 %.

In practice it is more convenient to add an equal volume of 40 % caustic soda to the solution than to weigh out the correct quantity of soda; in this way also a larger volume of liquid is contained in the flask. After boiling for six hours it is necessary according to van Slyke's directions to disconnect the parts of the apparatus and distil off the remaining ammonia which does not diffuse into the U-tube. Loss of ammonia may thus occur; it is sometimes noticeable by its smell. By using an equal volume of 40 % sodium hydroxide and consequently more liquid in the flask, it is not necessary to disconnect the parts of the apparatus; the ammonia remaining in the flask and condenser can be distilled directly into the U-tube if the water be run out of the condenser and the boiling continued for another 15 to 20 minutes.

The same results are obtained when the decomposition is carried out in a copper flask, but as will be seen from the data given by histidine under the same conditions it is not possible to use a copper flask.

(2) *Estimation of the Total Nitrogen of the Bases in the Solution.*

In van Slyke's procedure the total nitrogen of the arginine and of the other compounds in the solution is determined in the residue remaining after the arginine estimation. Owing to the presence of silica in the solution, dissolved by the alkali from the glass, bumping occurs and nitrogen is lost. This is lessened by using 20 % sodium hydroxide in the arginine estimation but is not eliminated; it is avoided by using a copper flask, but as previously stated a copper flask cannot be used.

It is advisable to estimate the total nitrogen in a separate portion (5 cc.) of the solution of the bases rather than in the residue.

(3) *Action of Caustic Alkali upon Histidine.*

The histidine used in these experiments was prepared by the usual method from defibrinated blood and isolated as hydrochloride. The preparation gave the following figures on analysis:

$$H_2O = 8{\cdot}55;\ N = 19{\cdot}8;\ Cl = 16{\cdot}7\ \%.$$

Calculated for

$$C_6H_9O_2N_3HCl\ .\ H_2O;\ H_2O = 8{\cdot}59;\ N = 20{\cdot}05;\ Cl = 16{\cdot}94\ \%.$$

20 or 25 cc. of approximately 0·5 % histidine hydrochloride solution were boiled with caustic soda of varying concentration in a glass or copper flask in the same way as described above in the case of arginine. The results were as follows:

(*a*) *With the copper flask.*

Concentration of NaOH %	Time of boiling hours	NH_3 evolved in cc. 0·1 N	Total nitrogen of histidine solution in cc. 0·1 N
50	6	3·3	14
50	6	4·6	23
25	6	1·9	13
25	11	2·1	13
20	8	1·9	13

(*b*) *With a glass flask.*

Concentration of NaOH %	Time of boiling hours	NH_3 evolved in cc. 0·1 N	Total nitrogen of histidine solution in cc. 0·1 N
50	2*	0·3	14·0
50	6*	0·3	14·0
50	6	0·4	23·0
20	6	0·5	13·0

* In these experiments the glass flask was destroyed by the alkali and the ammonia given in the table had diffused into the standard acid.

Histidine is thus decomposed by boiling with caustic alkali. The amount of decomposition in the cases in which a copper flask was used corresponds to about one-fourth of the total nitrogen of the histidine but the figures are not exact multiples and vary considerably. The amount of decomposition in the experiments in which a glass flask was used is very much less and corresponds to from 1·5 to 3 % of the total nitrogen in the solution.

This slight decomposition of histidine may however be neglected in the estimation of arginine; to some extent it makes up for the small loss which occurs in the determination of the arginine.

SUMMARY.

The estimation of arginine can be effected by boiling with 20 % sodium hydroxide, instead of 50 %, for six hours. The small loss of ammonia which occurs is counterbalanced by the slight decomposition of histidine which takes place when a solution of bases as obtained by van Slyke's method is being analysed.

The estimation cannot be effected in a copper flask if histidine is present, as under these conditions histidine undergoes considerable decomposition. It is advisable, on account of bumping with consequent loss of nitrogen, to estimate the total nitrogen of the arginine and other bases in a fresh portion of the solution rather than in the residue from the arginine estimation.

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XI. ON THE INOSITOL OF BRAIN AND ITS PREPARATION.

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Shortly after Scherer's [1850] discovery of inositol in muscle, von Bibra [1854] carried out a careful but unsuccessful search for this substance in brain. The presence of inositol in brain was first demonstrated by W. Müller [1857], and L. W. Thudichum [1884] is the only author who has since given attention to its occurrence.

The preparation of inositol from brain (and other organs) presents certain difficulties owing partly to the small amounts present and partly to the lack of trustworthy methods. The usual procedure, which has not varied much since Scherer [1850] first used it in the case of muscle, consists in extracting the organs with water and in precipitating the inositol from the suitably purified extract as an insoluble metallic compound by means of either basic lead acetate, copper acetate or baryta. From its combination with lead, copper or barium the inositol is set free by means of sulphuretted hydrogen or by sulphuric acid and finally crystallises out from the concentrated solution on the addition of alcohol.

W. Müller [1857] ground the brain with neutral lead acetate and water, allowed the mixture to stand and thus obtained a blood-red extract, which could be easily filtered and from which he was able to isolate inositol by means of basic lead acetate. The objection to this method is that it offers no guarantee of the complete extraction of the inositol, which moreover is usually obtained in a very impure condition in the first instance.

The difficulty encountered by von Bibra [1854] of filtering a watery extract of brain is easily overcome by gradually introducing the finely minced brain into boiling water and by acidifying with acetic acid before filtration. It

was to be expected that by a repeated extraction under these conditions practically the whole of the inositol would be removed. To my surprise however I found that when working up 1 kilo. of fresh ox-brain in this way, I was unable to obtain even a trace of inositol.

This result seems at first sight to be in favour of the view of F. Rosenberger [1908], according to which inositol does not occur as such in fresh organs, but is formed post-mortem by an autolytic or enzymic process from a precursor called "inositogen." This hypothetical substance was, however, not isolated as such and its existence was only inferred from the failure to isolate inositol itself. Rosenberger elaborated a method for the isolation of inositol from organs which is open to serious objections. He made use of the resistance of inositol to strong alkali in order to set it free from the tissues in a way similar to that used for the estimation of glycogen. The tissues are boiled with potash until completely dissolved and the solution is concentrated to one-third of its original volume after the addition of an excess of strong nitric acid. Hot baryta is next added and the heating continued for fifteen minutes, after which time the solution is again acidified and concentrated to a syrup. This is heated with concentrated nitric acid until the violent reaction is over, and again neutralised with baryta. If necessary the treatment with nitric acid and baryta is repeated several times. The final solution is cleared by neutral lead acetate and from the filtrate any inositol, which has survived this prolonged treatment, is precipitated by basic lead acetate in the usual way.

The negative results so often yielded by this method may well be due to the destruction and oxidation of inositol by the repeated treatment with strong nitric acid, as has been pointed out by E. Starkenstein [1908]. Moreover, the presence of the large quantities of salts introduced during the process may easily lead to the loss in the first lead precipitate of the small quantities of inositol normally present. It had already been noted by Thudichum [1884] that under certain conditions neutral lead acetate may carry down inositol and Meillère [1906] has traced this to the presence of salts.

It is not unlikely that von Bibra's and my own failure to isolate inositol from a brain extract made with boiling water is due to the same reason. I have not examined this subject any further, but as I was able to isolate inositol, by a method to be described, from the same lot of brain which failed to yield a trace by the usual extraction method, I incline to the view that as far as the brain is concerned the existence of "inositogen" is very unlikely.

I finally succeeded in finding a method which enables the preparation of inositol from brain to be made with certainty by making use of an observation kindly communicated to me by Dr O. Rosenheim. In the course of the method introduced by him [1906, 1914] for the preparation of lipoids from brain, acetone was used in the first instance for the dehydration of brain and the preparation of cholesterol. Rosenheim observed that the first watery acetone extract prepared at room temperature was free from cholesterol and that from it inositol could be easily prepared.

I was able fully to confirm this observation and found that this method of extraction of inositol leads more quickly to a pure product than W. Müller's original method and that the yield also is greater. I have not yet applied the method to any other animal organs or plants, but it is to be expected that it might be found useful in this case also, especially as the amount of inorganic salts, which interfere with the preparation of inositol, is considerably decreased when acetone is used as a solvent.

In his work on inositol, Thudichum [1884] suggests that the inositol from human brain "is either altogether different from that contained in the brain of the ox, or is accompanied by another similar carbohydrate of less stable quality." This conclusion was arrived at by the analysis of the copper compounds of a well-crystallised and apparently perfectly pure specimen of inositol from human brain, which contained up to 6 % more copper than the corresponding compounds prepared from bovine inositol.

This statement of Thudichum's, so many of whose originally discredited observations have since been confirmed, warranted further investigation. It is now known that inositol may exist in several optically isomeric modifications, although its accepted formula does not contain an asymmetric carbon atom. It was pointed out by O. Aschan [1902] that van't Hoff and Le Bel's theory allows the existence of seven inactive forms besides the two active ones. Of these *d*- and *l*-inositol are known to occur as methyl esters in plants, whilst the ordinary inositol of animals and plants represents one of the seven inactive forms. It has, however, recently been shown by Johannes Müller [1907] that a second inactive modification of inositol is represented by scyllitol, a substance discovered by Staedeler and Frerichs [1858] in the kidneys, liver and other organs of certain plagiostomous fishes (i.e. elasmobranchs such as the shark, dogfish, etc.). The same substance occurs also in acorns, originally called "quercine" by Vincent and Delachanal [1887] and in the leaves of coconut palms (*Cocos nucifera* and *Cocos plumosa*) from which it was isolated as "cocositol" by Hugo Müller [1907, 1912]. The two inactive

inositols resemble each other in their solubilities and chemical properties, but possess melting-points differing by as much as 125°.

I prepared inositol from brain by the method described above and from a comparison of their physical constants I came to the conclusion that the inositol occurring in human brain is identical with that of ox brain and the ordinary *i*-inositol. Thudichum's analytical figures therefore only prove that the copper compounds of inositol are of varying composition and not suitable for the identification of inositol.

Experimental.

(1) *The preparation of inositol from brain.*

Several attempts to isolate inositol by the usual methods from a brain extract made with boiling water were unsuccessful, not even enough material for a colour reaction being obtainable. Through Dr de Souza's kindness an acetone extract of 10 kilo. of ox-brain, which he did not require for the purpose of his work, was put at my disposal. This was freed from acetone by distillation and the remaining watery extract filtered. To the filtrate neutral lead acetate was added in excess. The resulting precipitate was filtered and washed. To the filtrate basic lead acetate and ammonia were added, the precipitate again filtered, suspended in hot water and decomposed with sulphuretted hydrogen. The lead-free filtrate was concentrated to a small bulk and alcohol added to the hot solution until crystallisation of inositol began. The crude inositol, which weighed about 4 g., was recrystallised from water and a well-crystallised product, showing a melting-point of 225°, was easily obtained. Having made myself familiar with the properties of inositol, I proceeded to compare the new acetone method with that of W. Müller's [1857] by which he originally obtained inositol from brain and with the method of Scherer [1850] which is successful in the case of muscle.

For this purpose a large quantity of fresh ox-brain was passed through a mincing machine. Three portions of 1 kilo. each were weighed out and worked up by different methods.

(*a*) *Müller's method.* One kilo. of minced brain was ground with solid lead acetate and one litre of water into a thin emulsion. After allowing the mixture to stand for about 18 hours a clear blood-red solution had separated from the brain mass, and this was filtered through muslin. The solution was boiled and filtered again. Basic lead acetate and ammonia were now

added, the bulky precipitate allowed to settle for some hours and filtered. According to my experience it is not advisable to wash this precipitate with distilled water, as the inositol-lead compound seems to dissociate on prolonged washing. It may, however, be washed with a solution of ammonium carbonate. The precipitate is next suspended in hot water and decomposed with sulphuretted hydrogen. The lead-free solution is concentrated to a small bulk (about 20–30 cc.) and absolute alcohol added until the crystallisation of inositol begins. After standing for 24 hours in the ice chest, the inositol is filtered and washed with alcohol and ether. The yield of crude, rather yellowish inositol was 0·22 g.

(*b*) *Scherer's method.* One kilo. of minced brain was gradually introduced into one litre of boiling water and acidified with acetic acid. After cooling a clear filtrate was easily obtained. The extraction with boiling water was repeated once more. The combined filtrates were precipitated with neutral lead acetate, the precipitate filtered and well washed. The filtrate from the neutral lead acetate was treated with basic lead acetate and ammonia as before. The final solution remained perfectly clear on the addition of absolute alcohol and I was unable to separate from it any substance giving the colour reactions typical for inositol.

(*c*) *Acetone method.* One kilo. of minced brain was well shaken up with one litre of acetone, left standing for about 18 hours and strained through several layers of muslin. The treatment with acetone was repeated, the combined extracts heated to boiling and filtered. From the clear yellowish filtrate the acetone was removed partly by distillation and finally, after the addition of water, on the water-bath. The watery solution was treated with lead acetate as before. The final lead precipitate was much less bulky than those obtained in the previous methods and the inositol, crystallising out on the addition of alcohol, was nearly white. The yield of crude inositol was 0·32 g.

(2) *The identity of the inositol of human and ox brain.*

A sample of inositol from human brain was prepared by the acetone method above described. If Thudichum's [1884] suggestion that the inositol of human brain is not identical with that of ox brain were correct, we should expect the inositol from human brain to represent either one of the optically active isomeric forms of inositol or another of the possible seven inactive forms. In both cases an examination of the behaviour towards polarised light and a determination of the melting-point

would form a sure criterion. The melting-point of scyllitol, the only other known form of *i*-inositol, is 350° [H. Müller, 1912], whilst ordinary inositol melts at 225°.

I examined 10 % solutions of inositol prepared from human and ox brain in a large Lippich-Polarimeter (with three field division) and failed to find in either case any optical activity.

The melting-point of two carefully purified specimens was 225°. A mixed sample showed no alteration of melting-point. Another sample mixed with *i*-inositol prepared from seeds (phytin) fused also at 225°.

Both preparations possessed the same solubilities and crystalline form and gave Salkowski's [1910] modification of Scherer's reaction, alike qualitatively as well as quantitatively.

The inositol of human brain agrees in its behaviour towards polarised light, in its melting-point and colour reaction with the inositol of ox brain, and both are identical with the ordinary *i*-inositol of plants.

The expenses of this investigation have been defrayed out of a grant made to Dr Rosenheim from the Government Grant Committee of the Royal Society.

Conclusions.

1. A trustworthy method for the preparation of inositol from brain, based on its solubility in dilute acetone, is described.

2. The inositol of human brain is identical with that of ox brain and with the ordinary *i*-inositol of plants.

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XII. ON THE OPTIMAL REACTION FOR PEPSIN.

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That the action of most ferments depends upon the hydrogen ion concentration is now a well-known fact. Sörensen [1909] was the first to study the optimal hydrogen ion concentration for pepsin. He made acid albumin solution from white of egg and, after digestion of it with pepsin, determined the increase of soluble nitrogen left after precipitation by tin chloride or tannic acid. Thus he found that in short durations of digestion ($\frac{1}{2}$ or 1 hour) the optimal hydrogen ion concentration was about $[H^{\cdot}] = 2{\cdot}3 \times 10^{-2}$, in longer durations (12 hours or longer) nearly $6{\cdot}0 \times 10^{-2}$, gradually moving to the acid side. Michaelis and Davidsohn [1910] used caseinogen as substrate and found the optimal concentration $[H^{\cdot}] = 1{\cdot}5 \times 10^{-2}$, with only short duration of digestion (10–15 minutes). Michaelis and Mendelssohn [1914] found, using edestin as substrate, that the optimal point of this ferment is, on the average, $4{\cdot}0 \times 10^{-2}$; concentrations between $2{\cdot}0 \times 10^{-2}$ and $8{\cdot}0 \times 10^{-2}$ being usually nearly the same in effect. They found further that the optimal hydrogen ion concentration does not depend upon the kind of acid nor on the presence of salts; in the latter case, it moves slightly to the less acid side.

Sörensen made all his experiments of the same chlorine concentration. In my experiments, the NaCl concentration was kept constant. The details are as follows:

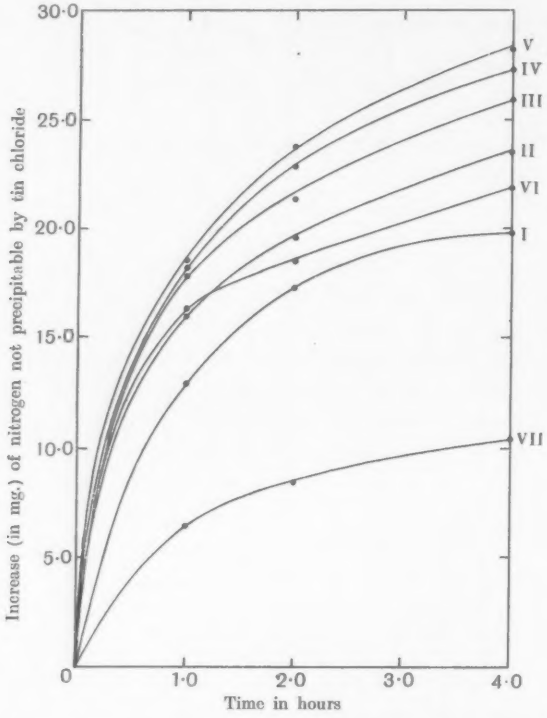
(1) 1 % *acid albumin solution as substrate.* About 3·3 % neutral protein solution was first made. This was obtained by mixing 600 cc. white of egg, 1146 cc. water and 54 cc. N/10 HCl. After shaking for two hours, this was put in the cold store until next day, when it was filtered. Then 1600 cc.

360 *cc. of approx.* 1 % *acid albumin solution* + 40 *cc. sodium hydrate solution containing sodium chloride* + 100 *cc. pepsin solution.* 30 *cc. of this mixture contained* 36·57 *mg. nitrogen, of which* 1·68 *mg. nitrogen was not precipitable by tin chloride,* 0·98 *mg. nitrogen not precipitable by tannic acid.*

Exp.	Composition of sodium hydrate + sodium chloride solution	Hydrogen ion concentration electrometrically measured		Increase (in mg.) of the amount of nitrogen which is not precipitable by tin chloride after t hours' digestion							Increase (in mg.) of the amount of nitrogen which is not precipitable by tannic acid after t hours' digestion						
		P_H	$[H^+]$	$t=\frac{1}{2}$	1	2	4	8	24	48	$t=\frac{1}{2}$	1	2	4	8	24	48
1	40 cc. 2N NaCl	0·53 (18° C.)	$3·0 \times 10^{-1}$	8·69	12·70	17·09	19·61	24·10	29·42	30·82	1·54	2·38	4·62	5·74	9·95	—	—
2	27 cc. 2N NaCl + 13 cc. 2N NaOH	0·74 (21° C.)	$1·8 \times 10^{-1}$	13·12	15·97	19·51	23·54	27·46	30·82	33·34	2·34	4·06	5·18	7·43	10·51	12·75	14·15
3	16 cc. 2N NaCl + 24 cc. 2N NaOH	1·24 (22° C.)	$5·8 \times 10^{-2}$	14·15	17·93	21·30	25·78	30·54	32·22	34·18	3·01	4·34	6·02	8·40	—	14·71	15·83
4	11 cc. 2N NaCl + 29 cc. 2N NaOH	1·37 (20° C.)	$4·3 \times 10^{-2}$	14·75	18·18	22·83	27·29	30·94	33·58	34·65	3·22	4·62	6·86	8·55	11·35	14·99	16·11
5	7 cc. 2N NaCl + 33 cc. 2N NaOH	1·76 (19·5° C.)	$1·7 \times 10^{-2}$	15·03	18·49	23·54	28·02	31·60	34·18	34·74	2·94	4·21	5·74	8·27	10·79	14·15	15·55
6	3 cc. 2N NaCl + 37 cc. 2N NaOH	2·22 (22° C.)	$6·0 \times 10^{-3}$	13·80	16·25	17·93	21·90	25·22	29·14	31·38	21·0	4·06	4·34	6·59	8·83	11·63	14·43
7	40 cc. 2N NaOH	4·15 (21° C.)	$7·1 \times 10^{-5}$	4·10	6·44	8·41	10·37	12·33	17·37	21·02	—	—	0·70	1·82	4·06	6·86	8·55

of this neutral protein solution were mixed with 2640 cc. water and 800 cc. N/1 HCl and the mixture put in an incubator (38°), and after 24 hours 30 cc. 5N HCl were added: 4 hours later 50 cc. 5N HCl were added and the mixture then kept another 24 hours at 38°.

(2) *Pepsin solution.* 10 g. scale preparation of pepsin was dissolved in 1000 cc. of 0·01N HCl; 10 cc. of this solution contained 1·6 mg. nitrogen.



For each experiment, as the table shows, 360 cc. acid albumin solution, 40 cc. of various mixtures of sodium hydrate and salt solution were mixed, and after warming to the temperature required there were added 100 cc. pepsin solution. The various solutions contained always the same salt concentration (about 0·16N NaCl). The digestion was carried out in an incubator at 38° C.

Precipitation with tin chloride or tannic acid solution was carried out in the manner of Sörensen. The hydrogen ion concentration of each test was electrometrically measured. It was found that the change of this

concentration during the course of digestion was quite small and could be neglected.

Controls were made by heating the pepsin in a water bath to 100° for one hour, so that the ferment was thoroughly destroyed. From these experiments it was found that the nitrogen not precipitated by tannic acid or tin chloride solution in the control was constant during the whole digestion. 30 cc. of control solutions contained at the beginning as well as at the end of digestion 0·98 mg. nitrogen not precipitated by tannic acid, and 1·68 mg. not precipitated by tin chloride, so that these values were subtracted from the values obtained in the experiments with the active enzyme.

The results are shown in the accompanying table and curves.

From the table it is to be seen that the increase in the amount of nitrogen not precipitable with tin chloride is greatest in Exp. 5, $[H^{\cdot}] = 1{\cdot}7 \times 10^{-2}$, then follow Exps. 4, $[H^{\cdot}] = 4{\cdot}3 \times 10^{-2}$, and 3, $[H^{\cdot}] = 5{\cdot}8 \times 10^{-2}$, showing no considerable difference between them. The increase of that not precipitable by tannic acid is most obvious in Exp. 4, then come Exps. 3 and 5. Therefore the optimum seems to be at about Exp. 4, or between Exps. 4 and 5.

Conclusion.

The optimal point for the action of pepsin is about $[H^{\cdot}] = 4 \times 10^{-2}$. Between $[H^{\cdot}] = 5{\cdot}8 \times 10^{-2}$ and $1{\cdot}7 \times 10^{-2}$ there is no considerable difference in the rate of hydrolysis.

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XIII. ON THE OPTIMAL CONDITIONS FOR THE PROTEOCLASTIC ACTION OF TAKA-DIASTASE.

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Introduction. Taka-diastase is prepared from koji-yeast and is chiefly used for the artificial digestion of amylaceous food. Koji-yeast itself is produced by the influence of *Aspergillus oryzae* on soaked rice. *A. oryzae* produces various kinds of ferments. Atkinson [1881] was the first to note that koji contains a strong diastase. Kellner, Mori and Nagaoka [1890] found that it converts starch into maltose and dextrin and, finally, into grape sugar; moreover, that it converts cane sugar into invert sugar. Saito [1906] reported that *A. oryzae* produces also trypsin, rennet, and catalase. Wohlgemuth [1912] found also erepsin, lipase and a haemolytic ferment. The conditions of the diastatic action of koji-yeast or taka-diastase have been studied by various authors. Kellner, Mori and Nagaoka observed that lactic acid above 0·1 % weakens the action of koji-diastase, but in a concentration of 0·05 % accelerates its action somewhat. They found also that it is strongly influenced in its diastatic or inverting action by the presence of salt, but does not completely lose its action even with 20 % of salt content. Saito also found this influence of salt in the case of pure cultures of the organism. According to Leo [1896], taka-diastase is not so sensitive to acids as is ptyalin, while Strauss and Stargard [1898] found no difference between them. Wohlgemuth confirmed Leo and even found the addition of dilute hydrochloric acid to accelerate the action of taka-diastase. With regard to its proteoclastic function, Wohlgemuth found on the contrary that taka-diastase is more active in media which are neutral or very slightly alkaline than in those which are very slightly acid.

The clinical use of taka-diastase was studied by Leo, and by Strauss and Stargard. These authors found that its amyloclastic function is alone of importance. So far as my experience goes, taka-diastase is very effective also in the digestion of proteins. This fact is not surprising, since Wohlgemuth found that 1 g. taka-diastase contains as much proteoclastic ferment as 100 cc. of human or animal pancreatic juice. This proteoclastic function seems more important than the amyloclastic and is of more clinical value, since the digestion of proteins is more frequently defective than that of carbohydrates. Consequently it is desirable to study the optimal conditions in which this ferment acts. As the proteoclastic ferment contained in taka-diastase is very similar in its action to that of pancreatic juice, I applied the method which Michaelis and Davidsohn [1911] used to study trypsin, with some modification in details. As ferment I used commercial taka-diastase which was always freshly dissolved to 10 % concentration. This solution, if kept 24 hours or longer, becomes black in colour, so that titration is interfered with, though it does not lose its activity. As substrate Witte's peptone was used, dissolved to 4 % in distilled water by boiling. It was filtered and kept sterile. The conversion was determined by Sörensen's formal method [1908]. For each experiment there were taken 10 cc. of regulator solution mixed with 30 cc. of 4 % peptone solution and, after warming to the required temperature, 10 cc. of 10 % taka-diastase solution were added. 10 cc. of digestion mixture were taken immediately after mixing and afterwards at any desired time, were put into a beaker containing 10 cc. of 40 % formal to stop digestion and then titrated with N/10 NaOH. Ten drops of 1 % phenolphthalein solution were used and a difference of less than 0·1 cc. could be detected, as Michaelis and Davidsohn observed with trypsin. The temperature was kept between 37–38° and several comparative estimations were made at the same time to control each other. The reaction of the digestion mixtures was measured by the electrometric method, which was carried out at the temperature of the room, mentioned in each case. Following the method employed by Michaelis and Davidsohn in their research on trypsin, I added such a quantity of regulators (mostly phosphate or acetate mixtures) as to neutralise the influence of the products of digestion on the reaction. Even in 24 hours no noticeable change occurred. The results are given in Table I.

From Table I it is to be seen that the optimum for the action of taka-diastase on Witte's peptone is at $[H^{\cdot}] = 8{\cdot}5 \times 10^{-6}$. This is shown also graphically in the figure.

With regard to the resistance of taka-diastase to acids Strauss and Stargard

TABLE I.

No.	Regulator mixture	[H·]	P_H	Time mins.	N/10 NaOH formal titration cc.
1	5·0 cc. N/3 Na_2HPO_4 ...	$9·1 \times 10^{-12}$	11·04 (19° C.)	20	0·10
	3·0 cc. N/1 NaOH ...			40	0·28
	2·0 cc. distilled water ...			60	0·39
2	5·0 cc. N/3 Na_2HPO_4 ...	$8·5 \times 10^{-9}$	8·07 (17·5° C.)	20	0·94
	1·5 cc. N/1 NaOH ...			40	1·74
	3·5 cc. distilled water ...			60	2·34
3	5·0 cc. N/3 Na_2HPO_4 ...	$8·9 \times 10^{-9}$	8·05 (19° C.)	20	1·08
	1·0 cc. N/1 NaOH ...			40	1·80
	4·0 cc. distilled water ...			60	2·40
4	5·0 cc. N/3 Na_2HPO_4 ...	$4·2 \times 10^{-8}$	7·38 (17·5° C.)	20	1·34
	0·5 cc. N/1 NaOH ...			40	2·02
	4·5 cc. distilled water ...			60	2·72
5	0·7 cc. N/3 NaH_2PO_4 ...	$9·1 \times 10^{-8}$	7·04 (19° C.)	20	1·42
	7·0 cc. N/3 Na_2HPO_4 ...			40	2·25
	2·3 cc. distilled water ...			60	3·10
6	Same as 5, but with boiled ferment			20	0
				40	0
				60	0
7	4·0 cc. N/3 NaH_2PO_4 ...	$4·0 \times 10^{-7}$	6·40 (16·5° C.)	20	1·62
	4·0 cc. N/3 Na_2HPO_4 ...			40	2·53
	2·0 cc. distilled water ...			60	3·35
8	4·5 cc. N/3 NaH_2PO_4 ...	$4·1 \times 10^{-7}$	6·39 (16·5° C.)	20	1·62
	4·0 cc. N/3 Na_2HPO_4 ...			40	2·65
	1·5 cc. distilled water ...			60	3·40
9	7·0 cc. N/3 NaH_2PO_4 ...	$1·1 \times 10^{-6}$	5·96 (17·5° C.)	20	1·67
	3·0 cc. distilled water ...			40	2·65
				60	3·40
10	1·0 cc. N/1 acetic acid ...	$2·1 \times 10^{-6}$	5·68 (19·0° C.)	20	1·70
	3·0 cc. N/1 sodium acetate			40	2·70
	6·0 cc. distilled water ...			60	3·50
11	1·5 cc. N/1 acetic acid ...	$4·3 \times 10^{-6}$	5·37 (17·0° C.)	20	1·75
	2·5 cc. N/1 sodium acetate			40	2·80
	6·0 cc. distilled water ...			60	3·60
12	2·0 cc. N/1 acetic acid ...	$8·5 \times 10^{-6}$	5·07 (17° C.)	20	1·73
	2·0 cc. N/1 sodium acetate			40	2·90
	6·0 cc. distilled water ...			60	3·65
13	Same as 12			20	1·78
				40	2·90
				60	3·67
14	Same as 12			20	1·75
				40	2·85
				60	3·65
15	Same as 12			20	1·84
				40	2·82
				60	3·62
16	Same as 12, but with boiled ferment			20	0
				40	0
				60	0

TABLE I (*continued*).

No.	Regulator mixture	[H·]	P_H	Time mins.	N/10 NaOH formal titration cc.
17	2·5 cc. N/1 acetic acid ...	$1·6 \times 10^{-5}$	4·80 (20° C.)	20	1·62
	1·5 cc. N/1 sodium acetate			40	2·56
	6·0 cc. distilled water ...			60	3·53
18	3·0 cc. N/1 acetic acid ...	$2·6 \times 10^{-5}$	4·59 (16° C.)	20	1·50
	0·3 cc. N/1 sodium acetate			40	2·30
	6·7 cc. distilled water ...			60	2·86
19	5·0 cc. N/3 NaH_2PO_4 ...	$1·9 \times 10^{-4}$	3·72 (15° C.)	20	0·95
	2·0 cc. N/1 HCl			40	1·61
	3·0 cc. distilled water ...			60	2·05
20	Same as 19, but with boiled ferment			20	0
				40	0
				60	0
21	5·0 cc. N/3 NaH_2PO_4 ...	$1·4 \times 10^{-2}$	1·84 (17° C.)	20	0·19
	5·0 cc. N/1 HCl			40	0·24
				60	0·29

found that the enzyme after one hour's contact with hydrochloric acid or gastric juice, of which the acidity is over 30 (0·11 %), loses entirely its amyloclastic power, so that no regeneration of its action in the intestine is to be expected when the medium resumes its neutral reaction. In respect to its proteoclastic function, my results are as follows:

Exp. 1. 1 g. taka-diastase was dissolved in 5·0 cc. N/10 HCl + 5·0 cc. N/10 NaOH. As regulator mixture was used 2·0 cc. acetic acid + 2·0 cc. sodium acetate + 6·0 cc. distilled water. Digestion was carried out in the way mentioned above.

Time mins.	Conversion N/10 NaOH cc.
20	1·75
40	2·93
60	3·75

Exp. 2. 1 g. taka-diastase was dissolved in 5·0 cc. N/10 HCl (at 19°). After one hour it was neutralised with 5·0 cc. N/10 NaOH. Other conditions the same as in Exp. 1.

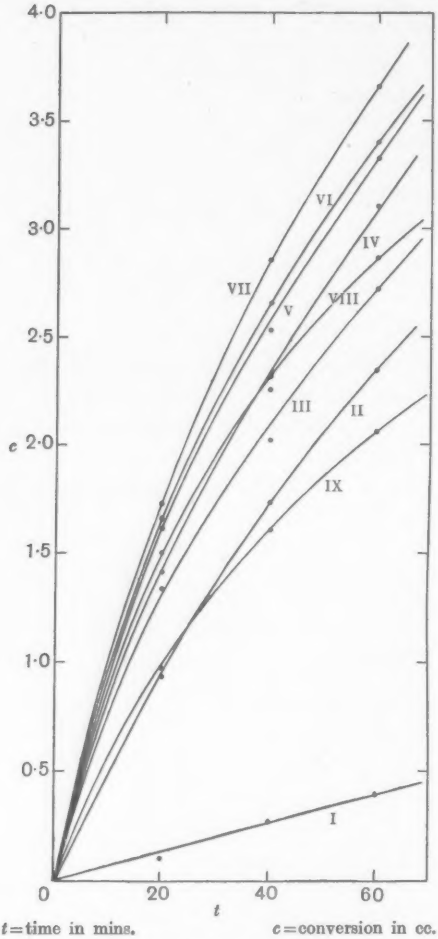
Time mins.	Conversion N/10 NaOH cc.
20	1·59
40	2·48
60	3·29

Exp. 3. Same as Exp. 2, only neutralised after two hours.

Time mins.	Conversion N/10 NaOH cc.
20	1·58
40	2·50
60	3·23

Exp. 4. 1 g. taka-diastase was dissolved in 5·0 cc. N/5 HCl + 5·0 cc. N/5 NaOH. Other conditions the same as in Exp. 1.

Time mins.	Conversion N/10 NaOH cc.
20	1·78
40	2·95
60	3·80



t=time in mins. c=conversion in cc.

Hydrogen ion concentrations.

I=$9{\cdot}1\times10^{-12}$ II=$8{\cdot}5\times10^{-9}$ III=$4{\cdot}2\times10^{-8}$ IV=$9{\cdot}1\times10^{-8}$ V=$4{\cdot}0\times10^{-7}$
VI=$1{\cdot}1\times10^{-6}$ VII=$8{\cdot}5\times10^{-6}$ VIII=$2{\cdot}6\times10^{-5}$ IX=$1{\cdot}9\times10^{-4}$

Exp. 5. 1 g. taka-diastase was dissolved in 5·0 cc. N/5 HCl. After one hour it was neutralised with 5·0 cc. N/5 NaOH. Other conditions the same as in Exp. 1.

Time mins.	Conversion N/10 NaOH cc.
20	0·73
40	1·33
60	1·60

Exp. 6. Same as Exp. 5, only neutralised after two hours.

Time mins.	Conversion N/10 NaOH cc.
20	0·70
40	1·22
60	1·55

Exp. 7. Same as Exp. 5, only neutralised after three hours.

Time mins.	Conversion N/10 NaOH cc.
20	0·65
40	1·10
60	1·45

From the above results one may judge that taka-diastase is not very sensitive to acid. During contact with hydrochloric acid below N/10 = 0·365 %, it loses its activity but little, on contact with N/5 HCl the diminution of its action is considerable; but this degree of acidity never happens in clinical cases. Longer or shorter duration of contact with acids, so far as relates to differences of one to three hours, has not so much influence as difference in degree of acidity. Hence, taka-diastase probably regains its action as soon as the medium resumes a suitable reaction. This justifies its clinical use.

As to the influence of salt on the proteoclastic function of taka-diastase, we see from the above results that 1 g. taka-diastase dissolved in 5 cc. N/10 HCl + 5 cc. N/10 NaOH or in 5 cc. N/5 HCl + 5 cc. N/5 NaOH has almost the same effect as that dissolved in 10 cc. distilled water, that is, taka-diastase dissolved in N/10 NaCl solution or in water has the same efficiency. Therefore the influence of salt in such concentrations is negligible.

Finally, it seems necessary to determine whether the results obtained apply to the tryptic or ereptic action. Wohlgemuth states that taka-diastase contains tryptic and ereptic ferments, like pancreatic juice; the action of the former is checked by blood serum while that of the other is accelerated

by it. Witte's peptone [Schjerning, 1898] contains, besides albumose, a certain amount of albumin; so that in the experiments given above, these two ferments may both have been in action. The following table seems to prove this fact.

TABLE II.

No.	Regulator mixture	P_H	Time mins.	Conversion N/10 NaOH cc.
1	2·0 cc. acetic acid ...	5·07	20	1·75
	2·0 cc. sodium acetate		40	2·85
	6·0 cc. distilled water		60	3·65
2	2·0 cc. acetic acid ...	5·12	20	1·70
	2·0 cc. sodium acetate		40	2·75
	1·0 cc. ox serum ...		60	3·46
	5·0 cc. distilled water		—	—
3	2·0 cc. acetic acid ...	5·10	20	1·40
	2·0 cc. sodium acetate		40	2·60
	2·0 cc. dog serum ...		60	3·05
	4·0 cc. distilled water		—	—

By adding blood serum to the regulator mixture a slight diminution of digestion was observed, though no important change in the reaction was produced. Since the diminution of the action is of a slight character, whereas that of trypsin itself should be strongly checked by serum, it seems reasonable to hold that the method used applies mainly to the ereptic function, the tryptic one being subsidiary.

Conclusions.

1. The optimal reaction for the proteoclastic ferments contained in taka-diastase is at $[H^{\cdot}] = 8{\cdot}5 \times 10^{-6}$, *i.e.* they act better in a medium of slightly acid than in one of neutral or slightly alkaline nature.

2. These ferments are considerably resistant to acid; so that they regain their activity after neutralisation, if the acidity does not exceed the degree which usually obtains in the stomach of man or animals.

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XIV. THE ESTIMATION OF REDUCING SUGARS BY KENDALL'S SOLUTION AND THE CONSTRUCTION OF A TABLE INDICATING THE REDUCING POWER OF LAEVULOSE.

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(*Received February 23rd, 1916.*)

While engaged in the estimation of sugars in plant tissues, we had occasion to employ Kendall's [1912] solution, consisting of copper sulphate, potassium carbonate, and salicylic acid. This was found by us to be extremely satisfactory and free from the possibility of error inherent in Fehling's solution, namely the solvent action of the hot sodium hydroxide upon the cuprous oxide. In all determinations Kendall's directions were carefully followed, but instead of estimating the copper reduced by this author's [1911] modification of the iodide method, or by the simpler routine adopted by Peters [1912], we proceeded in the following manner. The cuprous oxide was collected on asbestos in a Gooch crucible, washed with 360 cc. of hot distilled water (which quantity was found sufficient completely to remove the alkaline solution), then with a little alcohol and dried in a water oven. The crucible was placed inside a larger one and heated over a Teclu burner, as recommended by Davis and Daish [1913]. By heating in this manner for half an hour, all the cuprous oxide was converted into cupric oxide, and after allowing to cool in a desiccator for at least an hour, the crucible was weighed. The blue amphibole asbestos employed was treated with sodium hydroxide according to the well-known method of Munson and Walker.

By this method results were obtained for the reducing power of glucose, which were in close agreement with those in Kendall's table. Thus, 0·1345 g. of a sample of glucose, which had been recrystallised three times from 80 % alcohol and dried at 89°, gave 0·4745 g. of cupric oxide, the amount given in the table being 0·4743 g. This, it is worthy of note, was the first determination made by the authors by means of Kendall's solution.

Having thus satisfied ourselves that the method was reliable where pure sugars were concerned, it became necessary to ascertain whether the presence of citrates had any disturbing effect, since Davis and Daish [1913] have shown that citric acid inverts sucrose completely under conditions in which it does not hydrolyse maltose appreciably. Kendall himself tried to adapt Benedict's volumetric method to gravimetric sugar estimations, but came to the conclusion that the citrate in the solution employed, while dissolving but little cuprous oxide, had a depressing effect on the reducing power of the sugar. The following experiments show that it is not permissible to employ 2 % citric acid for the inversion of sucrose, when it is intended to estimate the products of hydrolysis by Kendall's method. Kahlbaum's sucrose from Indian cane was taken, the weight being corrected for the moisture present. The latter was determined by heating the finely powdered sugar in a closed vessel containing phosphoric oxide, the temperature being approximately 100°, till a constant weight was obtained.

A quantity of sucrose, 0·518 g., was made up to 100 cc. (solution *A*) with 2 g. of citric acid, and 20 cc. portions were heated for various times in a boiling water bath. Polarimetric readings showed that the change was not complete in fifteen minutes but was in thirty minutes.

For these observations and for the reductions the aliquot portions were made up to 25 cc. (solution *B*).

	CuO	
Solution *B*	20 min. heating	60 min. heating
20	0·3051 g.	0·3057 g.

From Kendall's table these values correspond to 0·495 g. sucrose per 100 cc. original solution. Thus it is clear that the citrate interferes seriously with the accuracy of the method. Accordingly it is advisable to employ invertase, free from maltase, for the inversion of sucrose in plant extracts since hydrochloric acid attacks laevulose and dextrose also to some extent.

Kendall's tables are constructed for glucose, invert sugar, lactose and maltose. As it was necessary for the purposes of our research to estimate laevulose with accuracy, a series of direct determinations of the reducing power of this sugar were made. In these experiments Kahlbaum's "laevulose from inulin I" was weighed out after prolonged drying in a desiccator. A portion of this very finely powdered was heated to the temperature of the water oven in a desiccating bottle connected with a tube containing phosphoric oxide, this tube being attached to an air-pump. After an hour of heating no loss of weight, whatever, was found to have occurred and the sugar was

therefore taken to be dry. The powder remained snow white throughout. Taking $[\alpha]_D^{20^\circ} = -92{\cdot}5^\circ$ for laevulose, a solution was found to contain 2·4760 g. by calculation from the rotation measured in a 20 cm. tube at 16·6° and corrected to 20°. The polarimeter used gave readings to a hundredth of a degree. As 2·4851 g. of laevulose had been weighed out the agreement was considered satisfactory and the laevulose was shown to be of a high degree of purity. The weights of the laevulose and of the cupric oxide thus obtained in the reductions were employed in the calculation of an equation of the form $y = a + bx + cx^2$, by the method of least squares. In this equation y denotes the weight of oxide and x represents that of the laevulose, both in grams. It should be noted here that the constants in Kendall's equation are intended for calculations in milligrams.

Evaluation of the constants gave the following figures:

$$y = 0{\cdot}0006 + 3{\cdot}910x - 2{\cdot}601x^2.$$

The observed and calculated values of y are recorded below.

x	y calculated	y found	Difference
0·0321	0·1234	0·1230	+0·0004
0·0400	0·1528	0·1533	−0·0005
0·0493	0·1871	0·1856	+0·0015
0·0777	0·2887	0·2914	−0·0027
0·0926	0·3404	0·3406	−0·0002
0·1285	0·4601	0·4611	−0·0010

From these figures it is clear that the equation yields values which agree fairly well with those found by direct experiment.

The accuracy is increased by the large amount of oxide obtainable by Kendall's method relatively to the amount of sugar employed. Thus the ratio CuO/laevulose varies from 3·83 to 3·58 in the above table.

Accordingly an error of 0·001 g. cupric oxide corresponds to less than 0·0003 g. laevulose. In the table which follows, the weights of oxide were calculated for every two milligrams of laevulose and the intermediate values interpolated. It may be added that for some as yet obscure reason it is not advisable to estimate laevulose by this method beyond the limits of concentration recorded in our table. Very numerous determinations were carried out at higher concentrations but the results obtained were not of such a degree of accuracy as to justify their use in constructing a table. We hope to study the point further.

In conclusion we wish to acknowledge our indebtedness to Prof. H. H. Dixon for all facilities for carrying out this work in the School of Botany, Trinity College, Dublin; the expenses were defrayed by the funds of the laboratory.

Laevulose mg.	Cupric oxide mg.	Laevulose mg.	Cupric oxide mg.	Laevulose mg.	Cupric oxide mg.
10·0	39·4	31·6	121·5	53·2	201·2
10·4	40·9	32·0	123·0	53·6	202·7
10·8	42·5	32·4	124·5	54·0	204·2
11·2	44·0	32·8	126·0	54·4	205·6
11·6	45·6	33·2	127·5	54·8	207·1
12·0	47·1	33·6	129·0	55·2	208·5
12·4	48·6	34·0	130·5	55·6	210·0
12·8	50·2	34·4	132·0	56·0	211·4
13·2	51·7	34·8	133·5	56·4	212·8
13·6	53·3	35·2	135·0	56·8	214·3
14·0	54·8	35·6	136·5	57·2	215·7
14·4	56·3	36·0	138·0	57·6	217·2
14·8	57·9	36·4	139·5	58·0	218·7
15·2	59·4	36·8	140·9	58·4	220·1
15·6	61·0	37·2	142·4	58·8	221·5
16·0	62·5	37·6	143·9	59·2	222·9
16·4	64·0	38·0	145·4	59·6	224·4
16·8	65·6	38·4	146·9	60·0	225·8
17·2	67·1	38·8	148·3	60·4	227·2
17·6	68·7	39·2	149·8	60·8	228·7
18·0	70·2	39·6	151·3	61·2	230·1
18·4	71·7	40·0	152·8	61·6	231·6
18·8	73·2	40·4	154·3	62·0	233·0
19·2	74·8	40·8	155·8	62·4	234·4
19·6	76·3	41·2	157·3	62·8	235·9
20·0	77·8	41·6	158·8	63·2	237·3
20·4	79·3	42·0	160·3	63·6	238·8
20·8	80·8	42·4	161·8	64·0	240·2
21·2	82·4	42·8	163·2	64·4	241·6
21·6	83·9	43·2	164·7	64·8	243·0
22·0	85·4	43·6	166·2	65·2	244·4
22·4	86·9	44·0	167·7	65·6	245·9
22·8	88·4	44·4	169·1	66·0	247·3
23·2	89·9	44·8	170·6	66·4	248·7
23·6	91·4	45·2	172·0	66·8	250·2
24·0	92·9	45·6	173·5	67·2	251·6
24·4	94·4	46·0	175·0	67·6	253·1
24·8	95·9	46·4	176·4	68·0	254·5
25·2	97·4	46·8	177·9	68·4	255·9
25·6	98·9	47·2	179·3	68·8	257·3
26·0	100·4	47·6	180·8	69·2	258·7
26·4	101·9	48·0	182·3	69·6	260·2
26·8	103·5	48·4	183·7	70·0	261·6
27·2	105·0	48·8	185·2	70·4	263·0
27·6	106·6	49·2	186·6	70·8	264·4
28·0	108·1	49·6	188·1	71·2	265·8
28·4	109·6	50·0	189·6	71·6	267·2
28·8	111·1	50·4	191·0	72·0	268·6
29·2	112·6	50·8	192·5	72·4	270·0
29·6	114·1	51·2	193·9	72·8	271·5
30·0	115·6	51·6	195·4	73·2	272·9
30·4	117·1	52·0	196·9	73·6	274·4
30·8	118·5	52·4	198·3	74·0	275·8
31·2	120·0	52·8	199·8	74·4	277·2

Laevulose mg.	Cupric oxide mg.	Laevulose mg.	Cupric oxide mg.	Laevulose mg.	Cupric oxide mg.
74·8	278·6	93·6	343·8	112·0	405·9
75·2	280·0	94·0	345·2	112·4	407·2
75·6	281·4	94·4	346·6	112·8	408·5
76·0	282·8	94·8	347·9	113·2	409·9
76·4	284·2	95·2	349·3	113·6	411·2
76·8	285·6	95·6	350·7	114·0	412·6
77·2	287·0	96·0	352·0	114·4	413·9
77·6	288·4	96·4	353·4	114·8	415·2
78·0	289·8	96·8	354·7	115·2	416·5
78·4	291·2	97·2	356·1	115·6	417·9
78·8	292·6	97·6	357·5	116·0	419·2
79·2	294·0	98·0	358·8	116·4	420·5
79·6	295·4	98·4	360·2	116·8	421·8
80·0	296·8	98·8	361·5	117·2	423·1
80·4	298·2	99·2	362·9	117·6	424·5
80·8	299·5	99·6	364·3	118·0	425·8
81·2	300·9	100·0	365·6	118·4	427·1
81·6	302·3	100·4	366·9	118·8	428·4
82·0	303·7	100·8	368·3	119·2	429·7
82·4	305·1	101·2	369·6	119·6	431·0
82·8	306·5	101·6	370·9	120·0	432·3
83·2	307·9	102·0	372·3	120·4	433·6
83·6	309·4	102·4	373·7	120·8	434·9
84·0	310·8	102·8	375·0	121·2	436·3
84·4	312·2	103·2	376·4	121·6	437·6
84·8	313·5	103·6	377·8	122·0	439·0
85·2	314·9	104·0	379·1	122·4	440·3
85·6	316·3	104·4	380·5	122·8	441·6
86·0	317·6	104·8	381·8	123·2	442·9
86·4	319·0	105·2	383·2	123·6	444·2
86·8	320·4	105·6	384·6	124·0	445·5
87·2	321·8	106·0	385·9	124·4	446·8
87·6	323·3	106·4	387·2	124·8	448·1
88·0	324·7	106·8	388·5	125·2	449·4
88·4	326·0	107·2	389·9	125·6	450·7
88·8	327·4	107·6	391·2	126·0	452·0
89·2	328·7	108·0	392·6	126·4	453·3
89·6	330·1	108·4	393·9	126·8	454·6
90·0	331·4	108·8	395·2	127·2	455·9
90·4	332·8	109·2	396·5	127·6	457·2
90·8	334·1	109·6	397·9	128·0	458·5
91·2	335·5	110·0	399·2	128·4	459·8
91·6	336·9	110·4	400·5	128·8	461·1
92·0	338·3	110·8	401·8	129·2	462·4
92·4	339·7	111·2	403·2	129·6	463·7
92·8	341·0	111·6	404·5	130·0	465·0
93·2	342·4				

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XV. THE GALACTOSIDES OF THE BRAIN. IV. THE CONSTITUTION OF PHRENOSIN AND KERASIN.

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In the first communication of this series [1913, 2] I expressed the view that the brain contains in a preformed condition mainly two representatives of the group of glucosides, namely the galactosides phrenosin and kerasin, which were originally discovered by Thudichum. A new method for their preparation was subsequently described together with a physical test (the selenite plate test) which furnishes a reliable method for controlling the efficacy of the methods used for their separation [1914, 1].

In the following communication are given the experimental data obtained by the chemical and physical examination of the galactosides together with the results of complete hydrolysis experiments.

The outcome of these experiments has been to establish for the first time the fact that kerasin possesses a laevorotation in distinction from the dextrorotatory phrenosin, and further that the only difference in chemical constitution of the two glucosides consists in their fatty acid component[1].

Whilst kerasin furnishes on hydrolysis inactive lignoceric acid ($C_{24}H_{48}O_2$), phrenosin gives rise to the optically active hydroxy-acid phrenosinic acid ($C_{25}H_{50}O_3$). The near relationship of these fatty acids is evident from the interesting fact that phrenosinic acid furnishes lignoceric acid on oxidation [Levene and Jacobs, 1912, 2]. Both glucosides yield the same carbohydrate, *d*-galactose, and the same base, sphingosine ($C_{17}H_{35}O_2N$), on hydrolysis.

These observations, together with the facts established by Levene and others with regard to the constitution of sphingosine, enable us now to construct constitutional formulae for these substances.

[1] The results of this work were communicated to the section of physiology of the 17th Internat. Congress of Medicine on Aug. 11th, 1913 [Rosenheim, 1913, 1]. Their publication in full has been delayed owing to unforeseen circumstances.

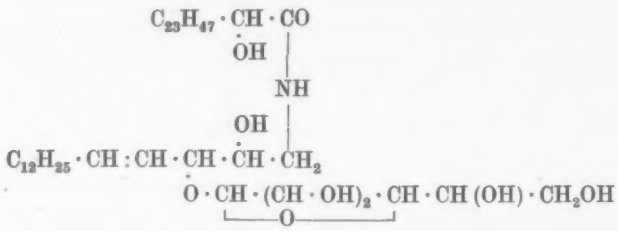
The unsaturated base sphingosine contains two free hydroxyl groups besides one amino-group [Levene and Jacobs, 1912, 1; Thierfelder and Thomas, 1912; Levene and West, 1914], and its formula may be written as: $C_{12}H_{25}CH:CH\cdot CH(OH)\cdot CH(OH)\cdot CH_2NH_2$. It is evident that in the galactosides the amino-group of the sphingosine is no longer free, since kerasin does not yield any nitrogen with nitrous acid when tested in van Slyke's apparatus. I was also unable to obtain any nitrogen from phrenosin by heating with hypobromite of potassium, and Levene and Jacobs [1912, 3] record incidentally a similar experience when testing their cerebrins in van Slyke's apparatus. We may therefore assume that in the galactosides the NH_2 group of sphingosine is in combination with the carboxylic group of the fatty acids, especially since the substances show neither any acid nor alkaline tendencies[1]. The possibility of a linkage of sphingosine by means of the NH_2 group with galactose must not be lost sight of. Up till now, however, no naturally occurring glucosides have been met with in which the carbohydrate is linked to nitrogen, and it is therefore much more likely that the aldehyde group of the galactose enters into combination with one of the hydroxyl groups of sphingosine. In analogy to the constitution of the other known naturally occurring glucosides, we may further conclude that phrenosin and kerasin are β-galactosides, in which galactose possesses the closed chain formation. From these considerations we arrive at the following constitutional formula of kerasin ($C_{47}H_{91}O_8N$):

$$C_{12}H_{25}CH:CH\cdot CH\cdot CH(OH)\cdot CH_2NH\cdot CO\cdot C_{23}H_{47}$$
$$\qquad\qquad\qquad\qquad\quad O$$
$$\qquad\qquad\qquad\qquad CH\cdot(CH\cdot OH)_2\cdot CH\cdot CHOH\cdot CH_2OH$$
$$\qquad\qquad\qquad\qquad\quad \llcorner\!\!\!-\!\!\!-\!\!\!-\!\!\!-O\!\!\!-\!\!\!-\!\!\!-\!\!\!-\lrcorner$$

This formula is in agreement with all the known facts of the reactions and cleavage products of kerasin. It allows for the formation of galactose, lignoceric acid and a salt of sphingosine during hydrolysis with a mineral acid in aqueous solution, whilst it also explains the formation of mono-methyl sphingosine and the methyl ester of lignoceric acid during methyl alcoholysis (see later). It contains five free hydroxyl groups which is in accordance with the formation of a penta-acetyl kerasin, a derivative prepared by Thierfelder [1914]. The position of the unoccupied hydroxyl group in the sphingosine residue in the above formula is arbitrary, since the relative position of the hydroxyl groups in sphingosine itself is still unknown.

[1] The tentatively advanced formulae [Rosenheim, 1913, 1] in which the presence of a free NH_2 group is assumed require therefore a modification in this direction.

The constitution of phrenosin is analogous to that of kerasin, the hydroxy-acid phrenosinic acid ($C_{25}H_{50}O_3$) taking the place of lignoceric acid. This is expressed by the following constitutional formula of phrenosin ($C_{48}H_{93}O_9N$):



We must assume that in the phrenosin molecule the two hydroxyl groups of sphingosine are at all events partially combined with other groups, since on alcoholysis besides sphingosine a certain amount of dialkyl sphingosine is formed. It is most probable that partial anhydride formation takes place between the sphingosine and phrenosinic acid hydroxyls. It is not likely that one of the hydroxyl groups of the galactose molecule is thus linked up, since on this assumption one would expect to find at least an indication of the formation of an alkyl galactose on alcoholysis. The linkage between the hydroxyl groups of phrenosinic acid and sphingosine is evidently only partial, since phrenosin furnishes on acetylation a hexa-acetyl derivative [Thierfelder, 1914]. This assumption also allows a satisfactory explanation for the existence of the two modifications of phrenosin. The ordinary "amorphous" phrenosin, which easily assumes the liquid-crystalline form [Rosenheim, 1914, 2], corresponds to the anhydric formula, whilst in the crystallised phrenosin the two hydroxyl groups are free. In accordance with this view is the fact that the crystallised modification contains one molecule of water more than the amorphous modification [Rosenheim, 1914, 2; see also Thierfelder and Wörner, 1900]. If this view is correct, the completely crystallised modification should give rise to a monoalkyl sphingosine on alcoholysis, a possibility which I have not yet tested experimentally. It may, however, be noted in this direction that Thierfelder and Thomas [1912] obtained on methyl alcoholysis of a sample of "cerebron" a substance which they considered at first as a monomethyl sphingosine. As the "cerebron" employed in this experiment consisted of a mixture of the crystallised and amorphous modifications, it is not unlikely that the cleavage product was a mixture of sphingosine with monomethyl sphingosine.

It is to be expected that the study of the products of a partial hydrolysis of these galactosides will furnish conclusive evidence to decide the correctness of the constitutional formulae advanced above, which may serve in any case as a basis for further research. Thudichum has already indicated the existence of two such compounds, which he called aesthesin (phrenosin minus galactose) and psychosin (phrenosin minus phrenosinic acid). The author is at present engaged in studying the action of enzymes on these galactosides. It is hoped that a suitable lipoclastic enzyme may split off the fatty acid component, leaving a sphingosine-galactoside, whilst an enzyme of the emulsin type may split off galactose and leave the sphingosine-fatty acid combination intact. The experiments made up till now show that phrenosin is resistant to pancreatic lipase and emulsin (commercial). An extract of brain from a case of extensive brain softening also had no action on phrenosin.

Experimental.

I. *Phrenosin.*

Elementary analysis. The analyses (1) and (2) refer to different samples of amorphous phrenosin prepared by the new pyridine method [Rosenheim, 1914, 1]. Analysis (3) was made of a sample of crystallised phrenosin [Rosenheim, 1914, 2]. The substances were dried at 105°.

(1) 0·1244 g.; 0·3183 g. CO_2; 0·1293 g. H_2O,
0·1293 g.; 2·3 cc. N at 26·0° and 714·5 mm.

(2) 0·1255 g.; 0·3207 g. CO_2; 0·1267 g. H_2O,
0·1321 g.; 2·2 cc. N at 26·0° and 714·5 mm.

(3) 0·1300 g.; 0·3305 g. CO_2; 0·1291 g. H_2O,
0·0960 g.; 1·7 cc. N at 17·5° and 722 5 mm.

	(1)	(2)	(3)	Average	Calc. for $C_{46}H_{93}O_9N$
C	69·78 %	69·69 %	69·34 %	69·60 %	69·65 %
H	11·63 „	11·29 „	11·11 „	11·34 „	11·24 „
N	1·90 „	1·79 „	1·91 „	1·83 „	1·70 „

Melting point. Phrenosin on heating assumes the liquid-crystalline condition, from a temperature of about 95° upwards until 210–212°, when it changes into the amorphous liquid condition. These phenomena have been fully described in a previous communication [1914, 2]. Phrenosin prepared by Thudichum's original method, as well as by that of Thierfelder, shows the same behaviour as phrenosin prepared by the pyridine method.

Optical activity. Phrenosin is dextrorotatory and the degree of its rotation is greatly influenced by the nature of the solvent, temperature and concentra-

tion. Pyridine is the most convenient solvent at ordinary temperatures and was first used in 1907 for this purpose by Rosenheim and Tebb in connection with some other work on brain lipoids. A 10 % solution of phrenosin in pyridine, however, possesses at 20° only about half the optical activity of a 5 % solution in chloroform-methyl alcohol at 40°, whilst a 10 % solution in the latter solvent at 45° shows nearly three times the optical activity of a 10 % pyridine solution at 20°. The estimations were made in a large Lippich polarimeter in a 1 dm. tube in sodium light.

Phrenosin (Pyridine method).

(1) 1·0046 g. in 10 cc. pyridine; $t = 20°$; $\alpha = +0·38°$; $[\alpha]_D^{20°} = +3·78°$.

(2) 1·0011 g. in 10 cc. pyridine; $t = 20°$; $\alpha = +0·37°$; $[\alpha]_D^{20°} = +3·70°$.

1·0000 g. in 10 cc. chloroform-methyl alcohol (3 : 1); $t = 45°$; $\alpha = +1·04°$; $[\alpha]_D^{45°} = +10·4°$.

Phrenosin (Thudichum's method).

1·000 g. in 10 cc. pyridine; $t = 18°$; $\alpha = +0·37°$; $[\alpha]_D^{18°} = +3·70°$.

1·000 g. in 10 cc. pyridine; $t = 30°$; $\alpha = +0·43°$; $[\alpha]_D^{30°} = +4·30°$.

0·500 g. in 10 cc. chloroform-methyl alcohol (3 : 1); $t = 40°$; $\alpha = +0·37°$; $[\alpha]_D^{40°} = +7·40°$.

Thierfelder and Kitagawa [1906] have previously found for "cerebron" $[\alpha]_D^{50°} = +6·4$ to $+8·4°$ in 5 % chloroform-methyl alcohol and Levene and Jacobs [1912, 3] for "*d*-cerebrin" (prepared by the baryta method) $[\alpha]_D^{25°} = +1·01$ to $+1·88°$ in pyridine. Later Levene [1913] has examined "cerebrin" fractions obtained by a process not yet fully described, for which he found $[\alpha]_D = +3·05$ to $+4·14°$ in pyridine and $[\alpha]_D = +9·5$ to $+10·7°$ in 6 % chloroform-methyl alcohol. My results agree, therefore, both with the earlier ones of Thierfelder and the later ones of Levene.

Hydrolysis. Many samples of phrenosin, prepared both by Thudichum's original method and by the pyridine method were hydrolysed. The products of hydrolysis were identical in both cases, qualitatively as well as quantitatively, namely (1) phrenosinic acid, (2) sphingosine and (3) galactose. Hydrolysis was effected by boiling under ordinary pressure (*a*) with dilute aqueous hydrochloric acid, or (*b*) with methyl alcoholic sulphuric acid. In the latter case the methyl-ester of phrenosinic acid and small quantities of dimethylsphingosine, in addition to sphingosine, are formed.

(1) *Phrenosinic acid* ($C_{25}H_{50}O_3$)[1]. 1·0 g. phrenosin (prepared by the

[1] Phrenosinic acid = Thudichum's "neurostearic" acid = Thierfelder's "cerebronic" acid. (See O. Rosenheim, 1913, 2.)

pyridine method) was hydrolysed by boiling its solution in 50 cc. methyl alcohol, containing 5 % sulphuric acid, for 5·5 hours on a reflux. The solution remained perfectly colourless and on cooling a crystallised white precipitate formed. The mixture of free fatty acid and ester was filtered off, washed with cold methyl alcohol and converted into the sodium salt by means of sodium methylate in methyl alcoholic solution. The sodium salt was recrystallised from boiling absolute alcohol and the acid liberated by dilute sulphuric acid. The free acid was taken up in ether and its ethereal solution washed with water in a separating funnel until free from mineral acid. The white fatty acid left on evaporation of the filtered ether solution weighed 0·305 g. and fused at 103°–104°.

Phrenosinic acid crystallises from alcohol in characteristic forms which Thudichum [1881] has described as "mamillary" or cauliflower-like masses. Under the polarising microscope they are seen to consist of a conglomerate of irregular spherocrystals, which behave in the selenite plate test [Rosenheim, 1914, 1] exactly as the mother substance phrenosin.

For analysis the acid was recrystallised three times from acetone. After the last recrystallisation and drying *in vacuo* it showed a melting point of 105°–106° and a dextrorotation of $[\alpha]_D^{20°} = +3{\cdot}86°$ in pyridine ($l = 1$; $c = 2{\cdot}849$; $t = 20°$; $\alpha = +0{\cdot}11°$).

On analysis it gave the following figures:

0·0792 g.; 0·2195 g. CO_2; 0·0894 g. H_2O.

	Found	Calc. for $C_{25}H_{50}O_3$
C	75·58 %	75·30 %
H	12·63 „	12·65 „

Sodium salt. Another preparation of phrenosinic acid was converted into the sodium salt, which was recrystallised from absolute alcohol and dried at 105°. 0·1042 g. gave 0·0172 g. Na_2SO_4;

Na = 5·39 %. Calculated for $C_{25}H_{49}O_3Na$ = 5·48 %.

Molecular weight estimation. A sample of phrenosin, prepared by Thudichum's method, was hydrolysed as above described. The ester was converted into the potassium salt and the free acid, prepared from it, recrystallised from acetone. It fused at 102°–103°. The acid was dissolved in benzene and titrated with 0·1N alcoholic potash (phenolphthalein as indicator). 0·3257 g. required 8·24 cc. 0·1N KOH.

Molecular weight = 395. Calc. for $C_{25}H_{50}O_3$: 398.

Another sample was hydrolysed by boiling for 24 hours with dilute hydrochloric acid. The mixture of free fatty acid and sphingosine hydrochloride was filtered after the addition of a few cc. of saturated sodium sulphate solution which renders quick filtration possible. The acid was extracted with ether and purified by means of the barium salt. The barium salt was decomposed with tartaric acid and the free acid extracted with ether. It fused at 102°–103°. 0·2687 g. required 6·73 cc. 0·1N KOH. Molecular weight = 399. Calc. for $C_{25}H_{50}O_3$: 398.

Melting point of phrenosinic acid. The fatty acid obtained by Thudichum from phrenosin and called by him "neurostearic" acid had a melting point of 84°, but the same elementary composition as phrenosinic acid. Thierfelder's "cerebronic" acid, prepared by a method almost identical with that of Thudichum, melted at 98°–99° but yielded on analysis practically the same figures as Thudichum's acid. Mainly on account of the difference in their melting points, Thierfelder [1905] declined to admit the identity of these acids and their mother substances. More recently, however, Levene and Jacobs [1912, 2] again obtained Thudichum's acid of the lower melting point and showed that "cerebronic" acid exists in two optically isomeric forms of which the inactive one melts at 82°–85° (Thudichum's "neurostearic" acid), whilst the dextrorotatory form melts at 106°–108° (Thierfelder's "cerebronic" acid). A mixture of the two forms melting at 92°–95° is frequently obtained [Rosenheim and MacLean, 1915]. The conditions under which these two isomers are formed are not yet explained. In some preliminary experiments I have found that the melting point of the dextrorotatory acid (103°–104°) is lowered considerably by keeping it for some time in a toluene bath. After six hours the melting point had fallen to 88°–89°. Experiments are in progress to decide whether this decrease goes hand in hand with a racemisation. The change in melting point may possibly be due also to the formation of an anhydride or lactone-like derivative owing to the presence of a hydroxyl group in phrenosinic acid. The difficulty mentioned by Levene and West [1913] of obtaining correct titration values of the inactive acid is in favour of such an explanation.

(2) *Sphingosine* ($C_{17}H_{35}O_2N$). This base was first obtained by Thudichum on hydrolysis of phrenosin with dilute sulphuric acid under pressure. The peculiar name was given to it "in commemoration of the many enigmas which it presented to the enquirer" [Thudichum, 1881]. Thierfelder [1904] confirmed Thudichum's results when he hydrolysed his "cerebron" by the same method; but later Thierfelder and Kitagawa [1906] expressed doubts

as to the uniformity of sphingosine, as they obtained on hydrolysis in methyl alcoholic solution a mixture of sphingosine with another base of the composition $C_{19}H_{39}O_2N$. These doubts were removed by Levene and Jacobs [1912, 1], who showed that the second base, dimethylsphingosine, was formed during alcoholysis and was not originally present in the phrenosin molecule. These observations were confirmed by Thierfelder and Riesser [1912].

I have obtained sphingosine from phrenosin (prepared by Thudichum's method) on hydrolysis with dilute hydrochloric acid, and both sphingosine and dimethylsphingosine from phrenosin (prepared by the pyridine method) on hydrolysis with sulphuric acid in methyl alcoholic solution. Only the identification of the latter need be described here.

The filtrate obtained above after the removal of phrenosinic acid and its ester was diluted with 100 cc. water and the alcohol evaporated on a water-bath. The solution became cloudy and a semi-solid white substance collected on the surface, which solidified on cooling. This was filtered off and dissolved on the filter in boiling alcohol. On evaporation of the alcohol and drying *in vacuo* the residue of sphingosine sulphate and dimethylsphingosine sulphate weighed 0·550 g. The two substances were separated by their different solubilities in alcohol.

(*a*) *Sphingosine.* The mixture of the sulphates was treated repeatedly at room temperature with alcohol. From the insoluble portion the base was set free by means of alcoholic potash. It was extracted with ether and the ether solution washed with water. On evaporation of the ether the base remained as a syrup, which crystallised completely in rosettes of needles. The base was dissolved in alcohol and reconverted into the sulphate by the addition of alcoholic sulphuric acid. The process of purification was repeated once more and sphingosine sulphate was obtained in perfectly white crystals, which were filtered, washed with ether and dried *in vacuo*. The salt is hygroscopic and melts with decomposition at 233°–235° (Levene and Jacobs give 233°–239°). Its solution in methyl alcoholic sulphuric acid showed a laevorotation of $[\alpha]_D^{18°} = -9{\cdot}47°$.

$$(l = 1; \quad c = 1{\cdot}585; \quad t = 18°; \quad a = -0{\cdot}15°.)$$

(*b*) *Dimethylsphingosine.* The easily soluble sulphate of dimethylsphingosine obtained above was converted into the free base and extracted with ether. The base crystallised at once on evaporation of the ether. It was converted into the hydrochloride by adding a methyl alcoholic solution of hydrochloric acid to its solution in methyl alcohol. The hydrochloride was

recrystallised from acetone, from which it separated in glistening plates. It melted at 133°–134° and gave the following results on analysis:

5·385 mg.; 12·875 mg. CO_2; 5·84 mg. H_2O.

	Found	Calc. for $C_{19}H_{39}O_2N \cdot HCl$
C	65·20 %	65·18 %
H	11·58 „	11·52 „

(3) *d-Galactose.* The solution from which phrenosinic acid and sphingosine had been removed, as above described, contained only *d*-galactose as the last product of hydrolysis. This was estimated quantitatively by means of the polarimeter. The solution was made up to 100 cc. and showed in a 2 dm. tube a rotation of $\alpha = +0{\cdot}35°$. Whence galactose = 21·63 %; calc. for 1 mol. galactose from $C_{48}H_{93}O_9N$ 21·77: %.

d-Galactose was identified by means of its characteristic methylphenylhydrazone. Solid sodium acetate was added until the reaction was no longer acid to Congo paper. A few drops of methylphenylhydrazine were added, and after standing at room temperature crystals of the hydrazone settled down. They were filtered off, washed with water and recrystallised from alcohol. The perfectly white crystals of the hydrazone melted sharply at 190°. Neuberg [1907] gives the melting point of galactose methylphenylhydrazone at 191°.

Comparison of the galactoside, prepared from brain by the pyridine method, with Thudichum's phrenosin shows their complete agreement in all points. It follows from this that Thierfelder's "cerebron," with which my substance agrees in every way, is also identical with phrenosin. Gamgee's "pseudo-cerebrin" is admittedly identical with "cerebron" prepared by Thierfelder's original method (1904) whilst there can be no doubt that the "cerebrin" of Parcus as well as that of Kossel and Freytag is identical with "cerebron" prepared by Thierfelder's later baryta method [1913].

Hence it follows that all these products represent one and the same substance, namely phrenosin. The unfortunate confusion which has been brought into the nomenclature of the brain lipoids by the introduction of new names for one and the same substance can now be considered to be cleared away experimentally. It seems only just to retain the name phrenosin introduced in 1874 by Thudichum, who discovered the substance and was the first to establish the nature of its cleavage products. This suggestion has previously been made, based on theoretical considerations, by Posner

and Gies [1906] and Rosenheim and Tebb [1907] and was adopted by myself in the proposals for the nomenclature of the lipoids [1909].

II. *Kerasin.*

Elementary analysis. The analyses (1)–(4) were made of different samples of kerasin prepared by the new pyridine method and sample (5) refers to kerasin prepared by Thudichum's original method, slightly modified [Rosenheim, 1914, 1]. The substances were dried *in vacuo* over sulphuric acid.

(1) 0·1363 g.; 0·3444 g. CO_2; 0·1395 g. H_2O,
0·1416 g.; 2·4 cc. N at 24° C. and 714 mm.
(2) 0·1317 g.; 0·3331 g. CO_2; 0·1372 g. H_2O.
(3) 0·1200 g.; 0·3034 g. CO_2; 0·1205 g. H_2O,
0·1379 g.; 2·3 cc. N at 25° C. and 714·5 mm
(4) 0·1185 g.; 0·2997 g. CO_2; 0·1187 g. H_2O,
0·1285 g.; 2·2 cc. N at 21° C. and 716 mm.
(5) 0·1156 g.; 0·2920 g. CO_2; 0·1184 g. H_2O,
0·1578 g.; 2·6 cc. N at 22·5° C. and 710 mm.

	(1)	(2)	(3)	(4)	(5)	Calc. for $C_{47}H_{91}O_8N+H_2O$
C	68·91	68·98	68·95	68·98	68·90	69·14 %
H	11·44	11·65	11·23	11·21	11·46	11·49 „
N	1·83	—	1·74	1·86	1·76	1·72 „

The values obtained are somewhat lower than those previously recorded in the literature. This may be due to the fact that the substances were dried *in vacuo* at ordinary temperature instead of at 100°–105°. The figures agree well with the theoretical formula $C_{47}H_{91}O_8N + H_2O$ which is calculated on the assumption that the kerasin molecule is composed of lignoceric acid ($C_{24}H_{48}O_2$), sphingosine ($C_{17}H_{35}O_2N$) and galactose ($C_6H_{12}O_6$) in equimolecular proportions.

It may be pointed out again that the elementary analysis is an unreliable index of the purity of these complex galactosides and does not prove the absence of phrenosin in kerasin preparations and *vice versa*. The difference in elementary composition between phrenosin ($C_{48}H_{93}O_9N$) and kerasin ($C_{47}H_{91}O_8N$) is so small that the presence of even a large percentage of phrenosin cannot be detected by elementary analysis. This is well illustrated by the experience of Levene and Jacobs [1912, 3], who originally considered phrenosin and kerasin as optical isomeric substances of the same chemical

composition, a view which seemed to receive support by the agreement of their elementary analyses. Further Thierfelder's [1913] substances of the kerasin type also yielded analytical figures which agreed well with the calculated formula and still it was found on hydrolysis that 25 % of the fatty acids consisted of the acid $C_{25}H_{50}O_3$, which is the characteristic component of phrenosin.

It is clear, therefore, that elementary analysis of these highly complex substances is of little value. Up till now the only satisfactory criteria of their purity are furnished by an examination of their optical activity and by the selenite test [Rosenheim, 1914, 1].

Melting point. The behaviour of kerasin on heating and the formation of liquid crystals has been fully described in a previous communication.

Optical activity. Kerasin is laevorotatory in distinction from the dextrorotatory phrenosin and the degree of its laevorotation may serve as an index for the more or less successful removal of phrenosin. Its optical activity increases with rising temperature and varies with the concentration and the nature of the solvent. The most highly purified specimens so far obtained showed an optical activity in pyridine at room temperature of $[\alpha]_D = -3{\cdot}71$ to $-3{\cdot}78°$. In a solution of chloroform containing 10 % pyridine, at 50°, their activity was $[\alpha]_D^{50°} = -4{\cdot}58$ to $-5{\cdot}08°$.

(1) 0·9132 g. in 10 cc. pyridine:
$t = 20°$; $l = 1$; $\alpha = -0{\cdot}25°$; $[\alpha]_D^{20°} = -2{\cdot}74°$.

(2) 0·9004 g. in 10 cc. pyridine:
$t = 18°$; $l = 1$; $\alpha = -0{\cdot}25°$; $[\alpha]_D^{18°} = -2{\cdot}78°$.

(3) 1·0000 g. in 10 cc. pyridine:
$t = 18°$; $l = 1$; $\alpha = -0{\cdot}25°$; $[\alpha]_D^{18°} = -2{\cdot}50°$.

(4) 1·0501 g. in 10 cc. pyridine:
$t = 25°$; $l = 1$; $\alpha = -0{\cdot}39°$; $[\alpha]_D^{25°} = -3{\cdot}71°$.
1·0044 g. in 10 cc. chloroform containing 10 % pyridine:
$t = 50°$; $l = 1$; $\alpha = -0{\cdot}51°$; $[\alpha]_D^{50°} = -5{\cdot}08°$.

Kerasin (Thudichum's method)[1].
1·0052 g. in 10 cc. pyridine:
$t = 25°$; $l = 1$; $\alpha = -0{\cdot}38°$; $[\alpha]_D^{25°} = -3{\cdot}78°$.
0·5025 g. in 10 cc. pyridine-acetone (1 : 1):
$t = 50°$; $l = 1$; $\alpha = -0{\cdot}23°$; $[\alpha]_D^{50°} = -4{\cdot}58°$.

[1] In connection with some work on the optical activity of brain lipoids Rosenheim and Tebb [1907] examined a sample of kerasin prepared by Thudichum's method and found its optical activity in a 3 % pyridine solution to be $[\alpha]_D^{18°} = -2{\cdot}8°$.

The kerasin fractions examined by Levene and Jacobs [1912, 3] were found by them to be inactive and they therefore proposed for kerasin the name *dl*-cerebrin (phrenosin = *d*-cerebrin). It may be pointed out, however, that they note that "once a sample was obtained from the kerasine fraction that was strongly laevorotatory." Thierfelder's [1913] kerasin fractions were also inactive or even slightly dextrorotatory. It is obvious from the above-mentioned presence of phrenosinic acid in them that they were contaminated with the dextrorotatory phrenosin to the extent of at least 12 %.

Molecular weight. Only one attempt has up till now been made to determine the molecular weight of kerasin by a physical method. Kossel and Freytag [1893] calculate from the rise of boiling point in glacial acetic acid solution the molecular weight of "homocerebrin" (= kerasin) as 986 (945, 1027). These figures can only be taken as approximate since it is well known that the accuracy of Beckmann's method is considerably impaired when applied to substances of high molecular weight which tend to form colloidal solutions. This may also be inferred by the experience of Pearson [1914][1]. Yet it seemed worth while to attempt the estimation of the molecular weight of kerasin by using Barger's [1904] microscopical method based on vapour pressure. Dr A. J. Ewins, who possesses considerable experience with Barger's method, kindly carried out the observations, for which I wish to take this opportunity of thanking him. Employing pyridine as a solvent and benzil as a standard, a solution of 0·080 g. kerasin in 0·500 g. of solvent was found to be between 0·20 and 0·22 mol., *i.e.* molecular weight lies between

$$\frac{0{\cdot}080 \times 1000}{0{\cdot}500 \times 0{\cdot}20} = 800 \text{ and } \frac{0{\cdot}080 \times 1000}{0{\cdot}500 \times 0{\cdot}22} = 727. \quad \text{Mean value} = 763.$$

[1] A chloroform solution of a mixture of galactosides, prepared by the baryta process, showed an elevation of the boiling point which stands in no relationship to the molecular weight. Pearson draws from his observations the conclusion that the galactosides occur in a chemical combination with sphingomyelin in the so-called "protagon." A single sample of "protagon," of which not even an analysis is given, was examined and failed to show any elevation of the boiling point, whilst a mixture of galactosides and sphingomyelin (prepared from "protagon" by the pyridine method) produced a measurable elevation. Quite apart from the probability that a similar measurable elevation might be found if several samples of "protagon" had been examined, the results recorded are to be expected, if only two components of the complex mixture "protagon" are examined by this method. The author failed to reconstitute the mixture "protagon" in its entirety, since he omitted to add the colloidal substances always present in protagon. These were removed by baryta in the one case and by the pyridine treatment in the other. It is admitted by Pearson that the contentions which were made in favour of the chemical entity of "protagon" were inconclusive and the whole case for "protagon" is now reduced to the value which can be attached to the few incomplete observations quoted. On the other hand the clear and experimentally uncontradicted chemical evidence has established the fact that "protagon" is a heterogeneous mixture.

Considering the size of the molecule, this figure agrees fairly well with the molecular weight ($C_{47}H_{91}O_8N = 797$) as calculated from the cleavage products of kerasin (see above).

It is interesting to note that in the case of phrenosin the method failed owing to the colloidal condition of sufficiently concentrated solutions.

Hydrolysis. This was carried out by the same methods as those employed for phrenosin previously described. Samples of kerasin prepared by the pyridine method as well as by Thudichum's method were hydrolysed and gave identical results. The products of hydrolysis were: (1) lignoceric acid, $C_{24}H_{48}O_2$; (2) sphingosine; and (3) galactose.

(1) *Lignoceric acid.* (*a*) *Methyl ester.* 3·50 g. of kerasin were hydrolysed by boiling under a reflux condenser for 6 hours with 250 cc. methyl alcohol containing 10 % conc. H_2SO_4. On cooling glistening white crystals of the methyl ester of lignoceric acid crystallised out which were filtered and dissolved in a small quantity of methyl alcohol. In order to complete the esterification HCl gas was passed into the hot solution and the ester, which crystallised out on cooling, was filtered, washed and dried *in vacuo*. It weighed 1·27 g. and was recrystallised in two fractions from acetone. The two fractions showed the same melting point of 57°–58°. According to Hell and Hermanns [1880] the methyl ester of lignoceric acid from wood paraffin melts at 56·5°–57° and Kreiling [1888] found the melting point 58° for the methyl ester of lignoceric acid from arachis oil.

The two fractions were combined and again recrystallised from acetone. The melting point remained unaltered. For elementary analysis the substance was dried *in vacuo* at 35°.

(9·075 mg.; 26·1155 mg. CO_2; 10·910 mg. H_2O.)

	Found	Calc. for $C_{23}H_{47}COOCH_3$
C	78·58 %	78·44 %
H	13·44 „	13·18 „

Molecular weight. 0·6044 g. of the ester was saponified by boiling for three hours with alcohol containing 20·42 cc. 0·1N KOH. The excess of potash was then titrated back by means of 0·1N HCl. 16·01 cc. of 0·1N KOH had been used up. Molecular weight = 382. Calc. for $C_{23}H_{47}COOCH_3$: 382.

Another sample of kerasin was hydrolysed in ethyl alcoholic solution. The ethyl ester of lignoceric acid was obtained and melted at 56°, solidifying

at 54°. Mixed with lignoceric acid ethyl ester from wood paraffin[1], it showed exactly the same melting and solidifying points.

(*b*) *Free lignoceric acid.* The potassium salt of lignoceric acid was obtained by saponification of the methyl and ethyl esters, filtered and well washed with alcohol and acetone. The fatty acid was set free from the soap by means of dilute hydrochloric acid and freed from mineral acid by repeatedly fusing its watery suspension on the water-bath. It was then extracted with ether, its ethereal solution washed with water, filtered through dry filter paper and evaporated. The white crystalline free acid was dried *in vacuo* and showed a melting point of 81°.

The acid was recrystallised twice from a mixture of light petroleum and acetone and was obtained in silky white crystals, forming a loose powder which assumed a wax-like consistency when compressed in an agate mortar.

Melting point. The substance had a sharp melting point of 81·0° and solidified at 74·5°–73·5°. The melting point of lignoceric acid is given in the literature as 80·5°–81° [Hell and Hermanns, 1880; Kreiling, 1888]. Samples were mixed with Hell's lignoceric acid and with lignoceric acid prepared by myself from arachis oil according to Kreiling. The melting points of the various mixtures were identical at 81·0°.

Optical activity. A 10 % solution of the acid in chloroform at 25° was found to be inactive.

Elementary analysis.

(1) 0·0745 g.; 0·2136 g. CO_2; 0·0862 g. H_2O.
(2) 5·145 mg.; 14·810 mg. CO_2; 5·848 mg. H_2O.

	(1)	(2)	Calc. for $C_{24}H_{48}O_2$
C	78·19 %	78·50 %	78·17 %
H	12·95 „	12·72 „	13·13 „

Molecular weight. (*a*) 0·2000 g. of the acid was titrated in light petroleum solution with 0·1N alcoholic potash (phenolphthalein as indicator) and required 5·39 cc. 0·1N KOH.

(*b*) 0·2205 g. of a different preparation (Thudichum's) required 5·97 cc. 0·1N KOH.

	(*a*)	(*b*)	Calc. for $C_{24}H_{48}O_2$
Molecular weight	371	369	368

[1] Through the kindness of Prof. Hell, the discoverer of lignoceric acid [1880], I came into the possession of samples of his original lignoceric acid and its ethyl ester, which also served for the identification of lignoceric acid from kidney lipoids [Rosenheim and MacLean, 1915].

The acid recovered from the potassium salts after titration showed the same melting point as before.

Silver salt. The acid was dissolved in alcohol and the silver salt precipitated by means of an alcoholic solution of silver nitrate. A drop of dilute alcoholic ammonia was added and the silver salt, which is not very sensitive to light, was filtered, washed with boiling alcohol and dried in a dark desiccator.

0·1208 g. gave 0·0273 g. Ag = 22·59 %,
Calc. for $C_{23}H_{47}COOAg$: 22·74 %.

Lead salt. The acid was dissolved in methyl alcohol and precipitated with a solution of lead acetate in methyl alcohol. A small amount of dilute methyl alcoholic ammonia was added to complete the precipitation. The lead salt was filtered, washed with hot acetone and dried *in vacuo*. It formed a white heavy powder which melted at 117°. The melting point of lead lignocerate is given as 117° by Hell and Hermanns [1880]. Another preparation was made from lignoceric acid obtained by the hydrolysis of Thudichum's kerasin.

(1) 0·1804 g. gave 0·0577 g. lead sulphate.
(2) 0·1508 g. gave 0·0482 g. lead sulphate.

	Pb	Calc. for $(C_{24}H_{47}O_2)_2Pb$
(1)	21·84 %	
(2)	21·83 „	21·99 %

(2) *Sphingosine.* After the removal of lignoceric acid the alcoholic hydrolysate was diluted with water, kept on the water-bath for some time and the alcohol finally evaporated. Just as in the case of phrenosin, it was found that besides sphingosine a certain amount of methyl derivatives of sphingosine had been formed during the methyl alcoholysis.

(*a*) *Sphingosine sulphate* was separated from this mixture by its relative insolubility in cold alcohol, in which methylsphingosine sulphate is soluble. After repeated purification by conversion into the free base, sphingosine sulphate was obtained as a white hygroscopic powder, showing a melting point of about 245°, and decomposing with effervescence at 250°. For further identification the amino-nitrogen was estimated by van Slyke's method, using the micro-apparatus recently described.

Amino-nitrogen estimation. A solution of sphingosine sulphate in glacial acetic acid was employed, containing 0·1484 g. of the substance in 10 cc. solution. 2 cc. were used for each experiment and the nitrogen evolution was found to be complete in 25–30 minutes. After making the necessary

correction, 2·01 cc. nitrogen at 763 mm. and 15° C. were obtained from 2 cc. of the solution.

	Found	Calc. for $(C_{17}H_{33}O_2NH_2)_2H_2SO_4$
N	3·96 %	4·19 %

(*b*) The *easily soluble sulphate* was converted into the free base by the addition of potash to its alcoholic solution. After addition of water the base was extracted with ether and it crystallised at once on evaporation of the solvent. The free base was dissolved in absolute ether and gaseous hydrochloric acid was passed into the solution. It solidified to a mass of crystals of the hydrochloride which was filtered off and washed with ether and acetone. The substance was recrystallised from a mixture of alcohol and acetone; it crystallised in transparent plates, resembling cholesterol crystals.

(0·0700 g.; 0·1644 g. CO_2; 0·0707 g. H_2O.)

	Found	Calc. for monomethylsphingosine hydrochloride	Calc. for dimethylsphingosine hydrochloride
C	64·05 %	64·32 %	65·18 %
H	11·30 „	11·40 „	11·52 „

The substance melted sharply at 141°, whilst dimethylsphingosine hydrochloride melts at 132°. The low carbon percentage and the higher melting point suggest that the substance represents a monomethylsphingosine hydrochloride.

In this connection it may be mentioned that Thierfelder [1913] also obtained on hydrolysis of various kerasin fractions a hydrochloride which showed the abnormal melting point of 139°. As the material employed for hydrolysis still admittedly contained phrenosin, it is possible that the substance, which was considered as dimethylsphingosine, consisted of a mixture of this substance with monomethylsphingosine. This question, which is of importance for the elaboration of the constitutional formula of kerasin, requires further investigation.

(3) *d-Galactose.* The carbohydrate contained in kerasin has up till now not been definitely identified. Thudichum assumed it to be identical with cerebrose (galactose) which he had obtained from phrenosin. I obtained the carbohydrate in the crystallised condition and identified it with galactose by oxidation to mucic acid and by its characteristic methylphenylhydrazone.

The hydrolysate of 3·50 g. kerasin, from which the fatty acid and bases had been removed as above described, was made up to 250 cc. and examined

polarimetrically. Actual rotation measured in 2 dm. tube = + 0·48°. Whence galactose = 21·19 %. Calc. for 1 mol. galactose from $C_{47}H_{91}O_8N$ = 22·6 %. Considering the unavoidable losses in the lengthy operations of the estimation, the value found agrees well with the calculated figure.

From one-half of the solution the carbohydrate was obtained by removing the sulphuric acid by means of barium carbonate and concentrating the filtrate *in vacuo*.

The residue was taken up in methyl-alcohol and the sugar recrystallised from water, from which it separated in the forms characteristic of galactose. A small quantity of the crystals was oxidised with dilute nitric acid according to Tollens. The mucic acid obtained showed the melting point of 190° and was converted into the ammonium salt by means of ammonium carbonate. The dry ammonium salt gave on heating a strong pyrrole reaction.

To the other half of the final hydrolysate solid sodium acetate was added until the reaction ceased to be acid towards Congo-paper. A few drops of methylphenylhydrazine were then added and the hydrazone separated and recrystallised as described above under phrenosin. The hydrazone showed a sharp melting point of 191°.

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Summary.

1. The galactoside phrenosin prepared from brain by the pyridine method is identical with Thudichum's phrenosin.

2. The substances described by various authors as pseudo-cerebrin cerebrin, cerebron and *d*-cerebrin are identical with phrenosin.

3. Phrenosin is dextrorotatory and furnishes on hydrolysis: (1) phrenosinic acid ($C_{25}H_{50}O_3$), (2) sphingosine, and (3) galactose.

4. Kerasin is laevorotatory and furnishes on hydrolysis: (1) lignoceric acid ($C_{24}H_{48}O_2$), (2) sphingosine, and (3) galactose.

5. Constitutional formulae for the two galactosides of brain are suggested.

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XVI. RECHERCHES SUR L'ANAPHYLAXIE PAR LA DIGLYCYLGLYCINE.

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INTRODUCTION.

Au cours de ses recherches sur la séroanaphylaxie du lapin, Arthus [1909, 1910] a soumis des lapins à 5 injections de 5 cm.^{3} chacune de solution aqueuse de glycocolle à 5 %. Quinze jours après la dernière injection, Arthus a introduit dans la veine auriculaire 4 cm.^{3} soit de sérum de cheval, soit de solution aqueuse de glycocolle à 5 %. Dans le premier cas, l'on obtient les réactions caractéristiques du choc anaphylactique, dans le second pas. Ces expériences mettaient en évidence deux faits très intéressants: 1° on peut anaphylactiser des lapins par des dérivés très éloignés des protéines; 2° une substance peut être anaphylactisante sans être toxique.

D'autre part, l'un de nous a montré [Zunz, 1911] que certaines protéoses (hétéroalbumose, protoalbumose, synalbumose) sensibilisent le cobaye et le lapin. Seules, l'hétéroalbumose et la protoalbumose déchaînent le choc anaphylactique chez les animaux préparés par l'une des trois protéoses précédentes. Chez le cobaye, le sérum de bœuf déchaîne encore plus aisément le choc que la protoalbumose ou l'hétéroalbumose, tandis que le sérum de cheval ne possède pas cette propriété.

Bien d'autres chercheurs se sont demandés, en ces dernières années, si l'on parvient à anaphylactiser les animaux au moyen de dérivés des protéines. Abderhalden a publié des résultats positifs [1914] obtenus chez des cobayes soumis à l'injection de polypeptides déjà assez compliqués. Mais Friedberger [1910; Friedberger et Joachimoglu, 1914] conteste les résultats d'Abderhalden et ceux de l'un de nous (Z.). Il estime qu'on n'est pas parvenu

jusqu'à présent à anaphylactiser des animaux par des protéoses, des peptides et, par conséquent, encore moins par des acides aminés.

Il serait trop long de passer en revue tous les travaux consacrés à cette question. Quoi qu'il en soit, en présence des résultats positifs obtenus par Arthus avec le glycocolle, on pouvait se demander si, en employant de la diglycylglycine ($NH_2.CH_2.CO.NH.CH_2.CO.NH.CH_2.COOH$), l'on n'arriverait pas à déchaîner au moyen de ce produit le choc anaphylactique chez des lapins préparés par des injections sous-cutanées de cette substance.

A cet effet, nous avons préparé, lors de chaque série d'injections, une solution fraîche de diglycylglycine à 2 % dans de la solution physiologique à 1 %. On a effectué une fois par semaine les injections préparantes sous la peau. Après trois à six injections, on a injecté dans la veine auriculaire du lapin la diglycylglycine ou le sérum frais de cheval huit jours après la dernière injection préparante.

On a enregistré la pression artérielle à l'aide d'un hémodynamographe de Verdin communiquant avec la carotide, la respiration à l'aide d'un tambour enregistreur de Marey, commandé par un pneumographe à double tambour appliqué sur la région diaphragmatique.

Pour apprécier la coagulabilité, nous avons rejeté les premiers cm.^{3} de sang carotidien souillés par mélange avec le liquide antihémostatique. Nous avons ensuite recueilli directement le sang de la carotide dans une capsule en porcelaine, sans lui permettre de couler sur la plaie. Nous avons noté le temps nécessaire, 1° à l'apparition des premiers flocons fibrineux, 2° à la coagulation en bloc.

Expériences.

I. *Effets de l'injection intraveineuse de diglycylglycine chez des lapins neufs.*

Il était nécessaire de reconnaître au préalable les effets éventuels de l'injection intraveineuse de diglycylglycine chez des lapins neufs. Nous résumons trois expériences de ce genre.

Expérience I. Un lapin, de 2300 gr., reçoit, en injection intraveineuse, 2·5 cm.^{3} de solution à 2 % de diglycylglycine, soit 5 centigr. de ce peptide. Ceci correspond à 2·15 centigr. de diglycylglycine par kilogramme.

La pression carotidienne s'élève d'abord légèrement; elle passe de 10·2 centimètres de mercure à 10·6, ½ minute après l'injection. Puis survient une chute graduelle et la pression atteint son minimum (8·4 centimètres) 14 minutes après l'injection pour ne plus remonter pendant les 10 minutes suivantes. Il s'est donc produit une chute de 1·8 centimètre de mercure.

La fréquence respiratoire, qui oscillait entre 42 et 55 mouvements par minute, a subi une légère accélération (60 à 74 mouvements par minute) de la dixième à la douzième minute consécutive à la fin de l'injection. A cette période, les mouvements respiratoires ont été peu amples. Ils ont ensuite repris le rythme initial.

Il ne s'est pas produit d'expulsion de bols fécaux.

Le sang carotidien, prélevé 25 minutes après la fin de l'injection, a montré les premiers flocons au bout de 20 minutes. La coagulation a été achevée 5 minutes plus tard.

Expérience II. Un lapin, de 1865 gr., reçoit, en injection intraveineuse, 10 centigr. de diglycylglycine, soit 5·3 centigr. par kilogramme.

A part une élévation passagère de 0·6 centimètre de mercure pendant l'injection, la pression artérielle s'est maintenue entre 9·8 et 10 centimètres de mercure.

Le rythme respiratoire rapide avant l'injection (82 mouvements par minute) subit un ralentissement graduel et se maintient entre 35 et 40 mouvements par minute, à partir de la quatorzième minute consécutive à l'injection.

Il y a eu expulsion de nombreux bols fécaux.

Le sang carotidien, prélevé 23 minutes après la fin de l'injection, a montré les premiers flocons au bout de 12 minutes. La coagulation complète a exigé 28 minutes.

Expérience III. Un lapin, de 1885 gr., reçoit, en injection intraveineuse, 10 centigr. de diglycylglycine, soit 5·31 centigr. par kilogramme.

L'élévation passagère lors de l'injection est plus accentuée ici que dans les expériences I et II. La pression carotidienne passe de 10 centimètres de mercure à 12·8, $\frac{1}{2}$ minute après l'injection. Puis survient une chute rapide de la pression revenue à son niveau initial 1$\frac{1}{2}$ minute après l'injection. La pression s'abaisse ensuite lentement et atteint son niveau le plus bas (9·2 centimètres de mercure) 21 minutes après l'injection. L'abaissement par rapport au niveau initial correspond alors à 0·8 centimètre.

Comme dans l'expérience II, le rythme respiratoire, fort rapide au moment de l'injection (94 par minute), subit un ralentissement graduel et ne représente plus que 35 mouvements par minute à la fin de l'expérience.

Il y a eu expulsion de 30 bols fécaux.

Le sang carotidien, prélevé 24 minutes après la fin de l'injection, a montré les premiers flocons au bout de 30 minutes. La coagulation complète a exigé 50 minutes.

Des trois expériences précédentes, il résulte que, par elle-même, l'injection intraveineuse de diglycylglycine a amené, deux fois sur trois, une chute peu accentuée et lente de la pression artérielle, n'atteignant pas 2 cm. de mercure. Le rythme respiratoire ne subit pas de modifications ou tend à se ralentir. La coagulation subit un retard. Il y a lieu de rapprocher ce dernier résultat des faits suivants établis par Zunz et György [1914] : Des doses très faibles de diglycylglycine accélèrent la coagulation du plasma oxalaté (de chien ou de lapin) recalcifié et parviennent même à faire coaguler du plasma insuffisamment recalcifié. Mais un excès de ce polypeptide entrave au contraire la coagulation du plasma oxalaté recalcifié. On ne doit donc pas interpréter le retard de la coagulation, à la suite de l'injection intraveineuse de diglycylglycine, comme un symptôme de choc anaphylactique.

II. *Effets de l'injection intraveineuse de diglycylglycine chez des lapins ayant subi au préalable des injections sous-cutanées de ce peptide.*

Passons maintenant aux expériences proprement dites. Recherchons tout d'abord les effets de l'injection intraveineuse de diglycylglycine chez des lapins préparés au moyen d'injections sous-cutanées de ce peptide,

répétées à sept jours d'intervalle. A titre d'exemple, nous résumons ci-dessous trois expériences ainsi effectuées.

Expérience IV. Un lapin, de 1825 gr., reçoit 3 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 9 centigrammes de ce peptide en 22 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en ½ minute, 5 cm.³ de solution à 2 % de diglycylglycine, soit 10 centigr. de ce peptide. Ceci correspond à 5·48 centigr. par kilogramme.

La pression artérielle s'abaisse graduellement de 10·8 centimètres de mercure avant l'injection à 8·2 quinze minutes après l'injection. La chute de pression atteint donc 2·6 centimètres à ce moment, où l'on a malheureusement interrompu l'expérience en injectant du sérum (voir plus loin, expérience XII).

On constate, en outre, une accélération passagère des mouvements respiratoires deux minutes après la fin de l'injection (80 par minute au lieu de 40 à 50 auparavant et par la suite). Ce sont là les signes d'une réaction anaphylactique nette, quoique peu intense.

Par contre, il n'y a pas eu d'expulsion de matières fécales.

Expérience V. Un lapin, de 2300 gr., reçoit 5 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 15 centigrammes de ce peptide en 36 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en ½ minute, 4 cm.³ de solution à 2 % de diglycylglycine, soit 8 centigrammes de ce peptide. Ceci correspond à 3·48 centigrammes par kilogramme.

La pression artérielle s'abaisse graduellement de 11·0 centimètres de mercure à 9·5. Cette chute maxima de 1·5 cm. est atteinte au bout de 13 minutes. La pression remonte lentement à partir de la seizième minute. L'abaissement de pression ressemble à celui observé dans l'expérience I chez un lapin non soumis à des injections préparantes sous-cutanées de diglycylglycine. En outre, le chiffre de mouvements respiratoires s'est maintenu pendant tout le cours de l'expérience entre 70 et 90 par minute. Par contre, il y a eu émission de bols fécaux.

Expérience VI. Un lapin, de 1915 gr., reçoit 6 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 18 centigrammes de ce peptide en 43 jours. Sept jours après la dernière injection sous-cutanée, on a introduit, dans la veine de l'oreille, en ½ minute, 5 cm.³ de solution à 2 % de diglycylglycine, soit 10 centigrammes de ce peptide. Ceci correspond à 5·22 centigrammes par kilogramme.

Il se produit une chute de pression atteignant 4·5 centimètres de mercure au bout de 16 minutes; la pression passe de 12·4 à 7·9 centimètres; elle remonte ensuite lentement. La respiration n'a pas subi de modifications appréciables. Aussitôt après l'injection, on a constaté, à travers la paroi abdominale, des mouvements péristaltiques très intenses des intestins, qui ont entraîné, au bout d'un quart d'heure, l'expulsion de 28 bols fécaux. Ce phénomène et l'intensité de la chute de pression permettent de conclure à une réaction anaphylactique nette.

Du sang carotidien, prélevé 20 minutes après la fin de l'injection, a montré les premiers flocons au bout de 25 minutes. La coagulation complète a exigé 30 minutes.

Les expériences IV et VI démontrent qu'il est possible d'anaphylactiser le lapin par des injections sous-cutanées de diglycylglycine. Trois injections pratiquées à sept jours d'intervalle suffisent déjà à cet effet, mais un plus grand nombre d'injections paraît préférable.

La diglycylglycine parvient à provoquer les symptômes du choc anaphylactique chez les lapins ainsi préparés.

Si l'on n'a pas obtenu de réaction anaphylactique dans l'expérience V, cela tient peut-être non pas à une quantité insuffisante d'injections préparantes, mais plutôt à la quantité trop faible de diglycylglycine introduite dans le sang.

Dans les expériences effectuées par Arthus avec le glycocolle, chaque lapin a été soumis à 5 injections de 5 cm.³ d'une solution aqueuse de glycocolle à 5 %, pratiquées sous la peau à sept jours d'intervalle. Il a donc reçu 125 centigrammes de glycocolle en 36 jours. Même en tenant compte de la différence entre les poids moléculaires de la diglycylglycine et du glycocolle et du plus grand degré de complexité moléculaire du premier de ces composés cristallins, on doit se demander, s'il n'y aurait pas eu intérêt, pour mieux mettre en évidence les phénomènes du choc anaphylactique, à utiliser de plus fortes doses de diglycylglycine, tant lors des injections sous-cutanées préparantes que lors de l'injection intraveineuse d'essai.

D'autre part, Arthus n'a jamais réussi à obtenir la moindre modification respiratoire ou circulatoire en injectant 25 centigrammes de glycocolle dans la veine auriculaire d'un lapin soumis à 5 injections préparantes de cet acide aminé. L'injection intraveineuse subséquente de sérum chez un tel animal démontre qu'il était néanmoins parfaitement anaphylactisé. Or, à la dose de 10 centigrammes, la diglycylglycine parvient déjà très bien à provoquer les phénomènes du choc anaphylactique. Nous nous trouvons donc en présence d'une différence essentielle entre le glycocolle et le peptide formé par l'union de trois molécules de cet acide aminé. Le glycocolle n'agit que comme sensibilisateur, alors que la diglycylglycine est à la fois sensibilisatrice et déchaînante.

III. *Effets de l'injection intraveineuse de sérum chez des lapins ayant subi au préalable des injections sous-cutanées de diglycylglycine.*

Résumons les résultats de trois expériences relatives à ce point.

Expérience VII. Un lapin, de 1930 gr., reçoit 5 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 15 centigrammes de ce peptide en 36 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en ½ minute, 10 centimètres cubes de sérum frais de cheval.

La pression artérielle s'abaisse, en 10 minutes, de 9·3 centimètres de mercure à 8 centimètres ; après cette chute de 1·3 centimètre, la pression remonte rapidement ; elle est déjà bien près du niveau initial 12 minutes après l'injection.

La respiration, fort rapide avant l'injection (110 mouvements par minute), se ralentit quelque peu après celle-ci (80 à 90 mouvements par minute).

Il y a en expulsion de 6 bols fécaux après l'injection.

Expérience VIII. Un lapin, de 2550 gr., reçoit 6 injections sous-cutanées de diglycylglycine. On lui a ainsi injecté en tout 18 centigrammes de peptide en 43 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en ½ minute, 5 cm.³ de sérum frais de cheval.

En 4 minutes, la pression tombe de 11 centimètres de mercure à 6·3 centimètres. Après cette chute de 4·7 centimètres, la pression remonte jusqu'à 9·4 centimètres, à la quatorzième minute consécutive à l'injection, pour redescendre de nouveau quelque peu ensuite et ne plus atteindre que 8 centimètres à la seizième minute et 8·1 à la dix-septième.

Avant l'injection, on compte 41 mouvements respiratoires par minute. Le nombre des mouvements s'élève fort vite et atteint 128 la deuxième minute après l'injection, 125 la troisième ; la respiration est donc devenue trois fois plus rapide qu'avant l'injection ; à ce moment, il y a de la dyspnée manifeste. Puis le rythme respiratoire revient vite à un chiffre oscillant entre 46 et 64 par minute.

Il n'y a pas eu d'émission de matières fécales.

Expérience IX. Un lapin, de 1980 gr., reçoit 6 injections sous-cutanées de diglycylglycine. On lui a injecté en tout 18 centigrammes de ce peptide en 43 jours. Sept jours après la dernière injection sous-cutanée, on introduit, dans la veine de l'oreille, en $\frac{1}{2}$ minute, 5 centimètres cubes de sérum frais de cheval.

Cinq minutes après la fin de l'injection, la pression artérielle est tombée de 10·8 à 7·6 centimètres de mercure, c'est-à-dire a subi une chute de 3·2 centimètres. La chute maxima n'est néanmoins atteinte qu'au bout de 18 minutes ; elle correspond à 4·2 centimètres de mercure.

Bien que le rythme respiratoire fût déjà très rapide (97 mouvements par minute) avant l'injection de sérum, celle-ci a cependant provoqué un accroissement notable de la fréquence des mouvements respiratoires. Cette accélération respiratoire a atteint son maximum (170 mouvements par minute) les quatrième et cinquième minutes après l'injection. La fréquence respiratoire a ensuite diminué ; elle correspondait à 125 mouvements par minute de la huitième à la dixième minute après l'injection, de 80 à 90 jusqu'à la quinzième minute, puis est descendue à 52 mouvements à la dix-huitième minute pour remonter à 70 à la dix-neuvième minute.

Il n'y a pas eu d'expulsion de matières fécales.

Du sang carotidien prélevé 20 minutes après la fin de l'injection, a montré les premiers flocons au bout de 16 minutes. La coagulation a été achevée 24 minutes après le prélèvement.

Les expériences VIII et IX faites chez des lapins soumis à 6 injections de diglycylglycine permettent de reconnaître que le sérum frais de cheval, introduit dans le torrent circulatoire de ces animaux, amène chez eux les symptômes du choc anaphylactique, tout comme chez les lapins préparés par des injections hypodermiques de glycocolle. Si la réaction est moins intense dans les expériences VIII et IX que dans les recherches effectuées par Arthus chez les animaux soumis au préalable à des injections de glycocolle, cela tient peut-être aux quantités relativement faibles de peptide reçues par nos lapins.

Le résultat négatif de l'expérience VII, effectuée chez un animal soumis seulement à 5 injections de diglycylglycine, tient peut-être à cette cause. Peut-être aussi la dose de sérum employée lors de l'injection intraveineuse d'essai (10 cm.^{3} au lieu de 5 dans les expériences VIII et IX) était-elle trop considérable. Enfin, il ne faut pas perdre de vue que, dans toutes les recherches relatives à l'anaphylaxie, on signale des animaux qui, pour un motif ou un autre, souvent ignoré de l'investigateur, ne réagissent pas comme la généralité des sujets en expérience.

Quoi qu'il en soit, les expériences IV à IX nous amènent à conclure qu'on parvient à anaphylactiser le lapin par un nombre suffisant d'injections sous-cutanées de diglycylglycine. On met aisément en évidence chez ces animaux la réaction anaphylactique par l'injection intraveineuse, soit de diglycylglycine, soit de sérum sanguin.

IV. *Effets de l'injection intraveineuse de diglycylglycine consécutive à l'injection intraveineuse de sérum chez des lapins ayant subi au préalable des injections sous-cutanées de diglycylglycine.*

A titre complémentaire, nous croyons utile de relater les deux essais suivants, dans lesquels des lapins, préparés par une série d'injections sous-cutanées de diglycylglycine, ont reçu, quelque temps après l'introduction de sérum frais de cheval dans le sang, une injection intraveineuse de diglycylglycine.

Expérience X. Dix-sept minutes après l'injection intraveineuse de sérum chez le lapin utilisé lors de l'expérience VII, lapin soumis au préalable à 5 injections sous-cutanées de diglycylglycine, on introduit, dans la veine de l'oreille, 5 cm.³ de solution à 2 % de diglycylglycine, soit 10 centigrammes de ce peptide. Ceci correspond à 5·19 centigrammes par kilogramme.

L'injection intraveineuse de diglycylglycine a amené, en 9 minutes, la pression artérielle de 9·3 à 7·5 centimètres de mercure. Cet abaissement, de 1·8 centimètre, est plus considérable que celui obtenu chez ce même lapin sous l'influence de l'injection intraveineuse de sérum (1·3 centimètre de mercure au bout de 10 minutes), mais il ne dépasse néanmoins pas celui obtenu dans l'expérience I sous l'influence d'une injection intraveineuse de diglycylglycine chez un lapin non soumis au préalable à des injections sous-cutanées de ce peptide. La pression artérielle remonte ensuite de 0·2 centimètre de mercure.

La respiration (110 mouvements par minute) s'est ralentie après l'injection de diglycylglycine ; elle a montré le minimum de fréquence, du reste encore assez élevé (65 mouvements par minute), 2 à 5 minutes après la fin de cette injection, puis elle a subi peu à peu une accélération qui l'a amenée, de la neuvième à la onzième minute consécutives à l'injection de diglycylglycine à un chiffre de 100 à 115 mouvements par minute pour redescendre ensuite au chiffre de 90 à 95 mouvements par minute.

Immédiatement après l'injection de diglycylglycine, il y a eu émission de 12 bols fécaux.

Du sang carotidien, prélevé 15 minutes après la fin de cette injection, a montré les premiers flocons au bout de 12 minutes. La coagulation a été achevée 2 minutes plus tard, soit 14 minutes après le prélèvement.

Expérience XI. Dix-huit minutes après l'injection intraveineuse de sérum chez le lapin utilisé lors de l'expérience VIII, lapin soumis au préalable à 6 injections sous-cutanées de diglycylglycine, on introduit, dans la veine de l'oreille, 5 cm.³ de solution à 2 % de diglycylglycine, soit 10 centigrammes de ce peptide. Ceci correspond à 3·92 centigrammes par kilogramme.

La pression artérielle a passé ½ minute après l'injection de diglycylglycine de 8·1 à 8·8 centimètres de mercure, puis est revenue à 8·1 centimètre. Il n'y a donc pas eu d'effet sur la pression artérielle.

Par contre, le nombre des mouvements respiratoires a passé de 52 par minute à 100, chiffre atteint 2 minutes après l'injection, puis est revenu à 60 la cinquième minute consécutive à l'injection.

Il n'y a pas eu d'émission de matières fécales.

Les expériences X et XI semblent montrer qu'une injection intraveineuse de diglycylglycine, consécutive à une injection intraveineuse de sérum, a les mêmes effets que celle-ci. On observe, dans les deux cas, soit une réaction anaphylactique (expériences VIII et XI), soit seulement une chute de la pression artérielle d'intensité insuffisante pour être considérée comme un symptôme du choc anaphylactique (expériences VII et X).

V. *Effets de l'injection intraveineuse de sérum consécutive à l'injection intraveineuse de diglycylglycine chez des lapins soumis au préalable à des injections sous-cutanées de ce peptide.*

Expérience XII. Quinze minutes après l'injection intraveineuse de diglycylglycine chez le lapin qui a servi à l'expérience IV, lapin soumis à 3 injections sous-cutanées préalables de ce peptide, on introduit, dans la veine de l'oreille, 5 cm.³ de sérum frais de cheval. L'injection intraveineuse de diglycylglycine avait amené les symptômes d'une réaction anaphylactique nette, mais peu intense.

L'injection de sérum fait monter la pression de 8·2 à 8·6 centimètres de mercure, puis l'abaisse, au bout de trois minutes, à 7·9 centimètres, niveau auquel elle reste ensuite.

Le nombre de mouvements respiratoires par minute s'abaisse de 54 à 44.

Il n'y a pas eu d'expulsion de matières fécales.

Du sang carotidien, prélevé 6 minutes après l'injection de sérum, a montré les premiers flocons au bout de 10 minutes. La coagulation a été achevée 5 minutes plus tard, soit 15 minutes après le prélèvement.

Expérience XIII. Dix-huit minutes après l'injection intraveineuse de diglycylglycine chez le lapin qui a servi à l'expérience V, lapin soumis à 5 injections sous-cutanées préalables de ce peptide, on introduit, dans la veine de l'oreille, 3 cm.³ de sérum frais de cheval. Quatre minutes plus tard, on injecte, dans cette même veine, 5 cm.³ de ce sérum. L'injection intraveineuse de diglycylglycine a amené, chez cet animal, une légère baisse de pression, sans accélération respiratoire concomitante.

La première injection de sérum a amené une légère élévation de la pression artérielle qui a passé de 9·9 à 10·5 centimètres de mercure. Le nombre de mouvements respiratoires s'est maintenu, comme avant l'injection, de 80 à 90 par minute. Il n'y a pas eu d'expulsion de matières fécales.

La seconde injection de sérum a fait monter la pression artérielle de 10·5 à 10·8 centimètres de mercure mais la pression est revenue de suite à 10·5 centimètres. La respiration a subi une accélération à partir de la troisième minute consécutive à l'injection ; elle a atteint son maximum de fréquence (120 mouvements par minute) les quatrième et cinquième minutes consécutives à l'injection ; l'accélération a persisté jusqu'à la dixième minute après l'injection.

Immédiatement après cette seconde injection, il y a eu expulsion de 69 bols fécaux.

Du sang carotidien, prélevé 12 minutes après la seconde injection de sérum, a montré les premiers flocons au bout de 20 minutes. La coagulation a été achevée 5 minutes plus tard, soit 25 minutes après le prélèvement.

L'injection intraveineuse de sérum effectuée quelque temps après l'injection intraveineuse de diglycylglycine a amené la réaction anaphylactique lorsque l'injection première de peptide ne l'avait pas produite (expérience XIII) et ne l'a pas entraînée au contraire lorsque l'introduction de diglycylglycine dans le sang l'avait déjà provoquée (expérience XII). Il se pourrait certes qu'on se trouve là en présence d'un simple hasard expérimental. On est néanmoins tenté d'admettre que le sérum n'agit que lorsque les effets de l'injection intraveineuse de diglycylglycine ont été nuls ou peu marqués.

RÉSUMÉ.

On parvient à anaphylactiser le lapin par des injections sous-cutanées de diglycylglycine, répétées à sept jours d'intervalle. Trois injections suffisent déjà parfois à cet effet, mais un plus grand nombre est préférable pour obtenir des résultats très nets.

L'injection intraveineuse, soit de diglycylglycine, soit de sérum frais de cheval, amène, chez les lapins ainsi préparés, les symptômes du choc anaphylactique : chute de la pression artérielle, accélération de la respiration, exagération des mouvements de l'intestin. Les divers symptômes ne coexistent pas dans tous les cas.

La chute de la pression artérielle doit dépasser deux centimètres de mercure pour représenter une réaction anaphylactique, car l'injection intraveineuse de diglycylglycine chez un lapin n'ayant pas été soumis à des injections préalables sous-cutanées de ce peptide amène parfois une chute peu accentuée et très lente de cette pression.

L'injection intraveineuse de diglycylglycine chez un lapin neuf ou bien n'entraîne aucun effet sur le rythme respiratoire ou bien tend à le ralentir.

La diglycylglycine ayant par elle-même une action sur la coagulation du sang, l'étude de la coagulabilité sanguine ne peut pas servir de critérium d'une réaction anaphylactique chez les animaux préparés par les injections sous-cutanées de ce peptide.

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